REMARKS

Please reconsider the application in view of the above amendments and the following remarks. Applicant thanks the Examiner for carefully considering this application.

Lack of Unity

Applicant confirms the election, without traverse, of Group I, claims 1-37, in which R^6, R^{6a}, R^7, R^{7a}, R^8, R^{8a}, R^9, and R^{9a} are each independently selected from hydrogen or C1-C3 alkyl, for continued prosecution.

Disposition of the Claims

Claims 1-37 are pending. Claims 1, 2, and 10 are independent claims. The remaining claims depend, directly or indirectly, from these independent claims.

Amendments to the Claims

Claims 1, 2, and 10 have been amended to restrict the limitations of the current claims to Group I, as elected by the Applicant, i.e., R^6, R^{6a}, R^7, R^{7a}, R^8, R^{8a}, R^9, and R^{9a} are each independently selected from hydrogen or C1-C3 alkyl. Claims 1-2, 4, 10-11, 19, 26 and 31 have been amended to clarify the recited inventions. Claim 1 has also been converted to a compound claim. No new matter is introduced by the amendments.

Claim Rejections under 35 U.S.C. § 112

Claims 1-9 and 36 are rejected under 35 U.S.C. §112 as not being enabling for a person skilled in the art to practice the invention regarding treatment of human diseases recited in these claims. This rejection is respectfully traversed.

Legal standard

The Examiner states that "the specification, while being enabling for inhibition of stearoyl-CoA desaturase activity in mice, does not reasonably provide enablement for inhibiting human stearoyl-CoA desaturase activity." (Office Action, p. 4). Applicants respectfully note

that testing on human is not a requirement for medical treatment claims using pharmaceutical compounds or compositions, nor is direct result showing treatment of the claimed diseases necessary. The proper inquiry is whether the tests/results described in the specification would be recognized by one skilled in the art as correlating with the claimed method invention.

"An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a 'working example' if that example 'correlates' with a disclosed or claimed method invention. . . . if the art is such that a particular model is recognized as correlating to a specific conditions, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications)." *See*, USPTO Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph – Enablement of Chemical/Biotechnical Applications, which can be found at http://0-www.uspto.gov.mill1.sjlibrary.org/web/offices/pac/dapp/1pecba.htm. (emphasis added).

"The evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art. A declaration or affidavit is, itself, evidence that must be considered. . . . The examiner should **never** make the determination based on personal opinion. The determination should always be based on the weight of all the evidence." *See*, USPTO Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph – Enablement of Chemical/Biotechnical Applications, cited above. (emphasis original).

That the evidence provided by applicant need not be conclusive is important because the nature of scientific inquiries often does not permit the scientist to make a definite conclusion. Typically, scientists make decisions, discoveries, and conclusions based on preponderance of evidence. As such, scientists typically cannot conclude that other possibilities do not exist. Therefore, the proper legal standard has always been whether one skilled in the art would be

convinced that a correlation exists between the data/model and the claimed methods/uses. That is, based on the knowledge of one skilled in the art, whether the evidence weighs "for" or "against" the correlation – i.e., based on preponderance of evidence. The Examiner should not substitute personal opinion for this determination. For reasons set forth below, Applicant respectfully submits that, in view of the prior art knowledge, a correlation exists between the disclosed information and the claimed methods.

The Specification teaches an *in vitro* assay for testing the claimed compounds' ability to inhibit stearoyl-CoA desaturase-1 activity. The assay is known in the art, see e.g., PCT publication No. WO 2001/062954. In addition to results described in the specification, further results for additional compounds in the claims (IC_{50} data) are submitted herewith (see attached 37 C.F.R. § 1.132 Declaration). As shown by these results and those described in the specification, the claimed compounds of the present invention exhibit activities as SCD inhibitors. In view of the state of the art at the time the instant application was filed (see discussion below), one skilled in the art would reasonably expect that the compounds of the invention, would be useful in treating diseases mediated by SCD, including type II diabetes, obesity, dyslipidemia, metabolic syndrome and acne in humans.

In one aspect, Applicants respectfully submit that one skilled in the art would reasonably expect that the compounds of the invention would be useful in treating type II diabetes by virtue of their ability to inhibit stearoyl-CoA desaturase activity. It is a well-known fact that patients with type II diabetes produce insulin, but lose the ability to respond to insulin signaling, i.e., the patients have decreased insulin sensitivity. Through the inhibition of stearoyl-CoA desaturase-1 activity, one can increase insulin sensitivity, thereby preventing or treating Type II diabetes. In Ntambi J.M. *et al., Proc. Natl. Acad. Sci.*, (August 20, 2002), Vol. 99, No. 17, pp. 11482-6, it was shown that mice with disrupted stearoyl-CoA desaturase-1 activity have increased insulin sensitivity (see first paragraph, pp. 11482). Moreover, it was shown (on page 11484) that stearoyl-CoA desaturase-1 knock-out mice showed improved glucose tolerance and a greater response to glucose lowering effect of insulin when compared to wild-type mice. The data supports the conclusion that inhibition of stearoyl-CoA desaturase-1 activity would lead to increased insulin sensitivity, which is a desired endpoint for the treatment of Type II diabetes.

Thus, Applicants respectfully submit that the instant application is enabling for the use of the compounds in treating humans for type II diabetes and/or for increasing insulin sensitivity.

In addition, Applicants respectfully submit that it is possible to treat obesity by inhibiting stearoyl-CoA desaturase-1 activity. As noted in Park, E.I. *et al., J. Nutr.* (1997), Vol. 127, pp. 566-573 (submitted herewith), mice provided with a diet that lowered the expression of stearoyl-CoA desaturase-1 had lower body weight and lower serum concentrations of total cholesterol, triglycerides, and HDL cholesterol. Furthermore, Ntambi *et al*, cited above, demonstrated that loss of stearoyl-CoA desaturase-1 function (activity) protected mice from gaining weight from a high-fat diet. Thus, one skilled in the art, in view of these references, would reasonably expect the compounds of the invention, by virtue of their ability to inhibit stearoyl-CoA desaturase-1 activity, to be useful in treating obesity in humans based on the disclosure of the Specification and the IC_{50} data.

With respect to treating dyslipidemia and lowering triglyceride, LDL and VLDL serum levels, WO 2001/062954 disclosed an animal model for testing the claimed compounds' effectiveness in lowering triglyceride, LDL and VLDL serum levels (see Example 1) and demonstrated the correlation between stearoyl-CoA desaturase-1 activity in humans and levels of serum triglycerides (see Example 2). Furthermore, as noted by Miyazaki, M. *et al., Journal of Lipid Research* (2001), Vol. 42, pp. 1018-1024 (submitted herewith), triglyceride synthesis was dramatically reduced in the liver of SCD -/- mice fed a lipogenic diet compared to normal mice. See also Miyazaki, M. *et al., J. Biol. Chem.* (2000), Vol. 275, No. 39, pp. 30132-30138 (submitted herewith). These observations demonstrated that the induction of triglyceride synthesis is highly dependent upon the expression of the stearoyl-CoA desaturase-1 gene. Thus, one skilled in the art, having knowledge of WO 2001/062954, the Miyazki references, the instant Specification and the IC_{50} data of the claimed compounds, would reasonably expect the compounds of the invention to be useful in lowering triglyceride, LDL, and VLDL serum levels and treating dyslipidemia in a human subject. Furthermore, as noted in Attie, A.D. *et al., Journal of Lipid Research* (2002), Vol. 43, pp. 1899-1907, which was published on August 16, 2002, stearoyl CoA desaturase activity was hypothesized as rate-limiting in triglyceride production in a wide array of dyslipidemias.

The Specification of the instant application provides a definition for the term "metabolic syndrome" on page 38, lines 27-31. The term "metabolic syndrome" is a recognized clinical term and has been used to describe a condition comprising at least one of type II diabetes, impaired glucose tolerance and insulin resistance, together with at least two of the following maladies hypertension, obesity, hypertriglyceridemia, low HDL or microalbuminemia. In other words, the term "metabolic syndrome" is used to describe a cluster of metabolic abnormalities. Disorders like "dyslipidemia, hypertension and obesity" are merely components of the metabolic syndrome and inhibition of stearoyl-CoA desaturase-1 activity can be a therapeutic treatment for each of these disorders individually or collectively. Given that it is a known fact that stearoyl-CoA desaturase-1 is a key regulator of fatty acid metabolism and insulin action (see Ntambi, J.M. *et al.*, *Journal of Lipid Research* (1999), Vol. 40, pp. 1549-1558, submitted herewith), a compound that inhibits stearoyl-CoA desaturase-1 activity can impact more than one component of the metabolic syndrome and will be useful in its treatment.

Furthermore, Applicants respectfully submit that it is possible to treat acne by inhibiting stearoyl-CoA desautrase-1 activity. Zheng *et al.*, *Nat. Genet.* (1999) 23:268-270 (submitted herewith), showed that rodents lacking a functional SCD1 gene had changes to the condition of their eyes, skin and coat thereby reducing the excessive sebum production that typically results in the formation of acne. As noted by Miyazaki *et al.*, J. Nutr. (2001), Vol. 131, pp 2260-68 (submitted herewith), SCD1-/- mice developed cutaneous abnormalities and atrophic sebaceous and meibomian glands compared to normal mice. These observations demonstrated that reduction of the sebum production can be effected by the inhibition of SCD1 and one skilled in the art would reasonably expect the compounds of the invention, by virtue of their ability to inhibit stearoyl-CoA desaturase-1 activity, to be useful in treating acne in humans.

Therefore, in view of the foregoing remarks, the Applicants respectfully submit that the Specification, in view of the existing knowledge in the prior art and the IC_{50} data, is clearly enabling for methods of treating disease mediated by SCD, including type II diabetes, obesity, dyslipidemia, metabolic syndrome and acne by the administration of a compound of the invention to the human.

1. Breadth of claims

a. Scope of the compounds

Applicant respectfully notes that methodology for the synthesis of these compounds is well within the skills of an ordinary artisan. Further, the specification, e.g., pages 49-76, discloses various synthesis schemes with examples of detailed synthesis.

b. Scope of the diseases covered

Applicants respectfully submit that one skilled in the art would reasonably expect that the compounds of the invention would be useful in treating various diseases mediated by SCD. Diseases mediated by SCD are not of unknown scope. As noted above, prior art is replete with evidence that inhibition of SCD can be effective in treating diseases mediated by SCD. One skilled in the art would not doubt that other diseases mediated by SCD can also be controlled by the inhibition of SCD. Applicants respectfully note that there is no need to demonstrate every single species included in a genus.

"For a claimed genus, representative examples together with a statement applicable to the genus as a whole will ordinarily be sufficient if one skilled in the art – in view of level of skill, state of the art and the information in the specification – would expect the claimed genus could be used in that manner without undue experimentation." (*see* the USPTO Training Materials cited above).

2. The nature of the invention and predictability in the art

Examiner cites Dobrzyn, et al. as stating, "However, the potential use of an SCD inhibitor as a human therapeutic agent awaits a more complete understanding of the mechanism underlying the effects of SCD deficiency and indication that the inhibition of this enzyme is both safe and efficacious."

Complete understanding of the mechanism and the safety is desirable, but not a criterion for patent protection. Furthermore, safety and efficacy issues are FDA

requirements, not PTO requirements. *See, Scott v. Finney*, 34 F.3d 1058, 1063 (Fed. Cir. 1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings."); *see also*, *In re Brana*, at 1567.

Applicants respectfully note that Dobrzyn does not really cast doubt on the rationale of using SCD inhibitors to treat SCD-mediated diseases. As noted above, there are plenty of prior art references supporting the notion of using SCD inhibitors as therapeutic agents of SCD-mediated diseases. In light of overwhelming evidence, Examiner should not rely too much on Dobrzyn. *See* the USPTO Training Materials cited above ("Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition").

More importantly, Sjogren, *et al.* (*Diabetologia*, Nov. 2007; e-publication ahead of print; the Abstract is attached) have confirmed a relationship between SCD activity and obesity/insulin resistance, previously observed in rodents, in humans. This publication proves that majority of the skilled artisans are correct and that Dobrzyn's doubt, if any, is unjustified.

3. Direction of guidance

The present Specification provides a general guidance for preferred dosage range (page 46, lines 17-25) and modes of administration (page 46, lines 26-29). A person with ordinary skill in the art who will be using these therapeutic agents is probably a researcher, a pharmacist or a physician. These people would know how to determine the optimal dosage and the optimal method of administration for treating SCD-mediated diseases in humans. Applicants respectfully submit that there is no need to include detailed information as to how to find the optimal dosages and how to administer these dosages to a patient.

"The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). A patent need not teach, and preferably omits, what is well known in the art. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986); *see also*, MPEP § 2182.

4. State of the prior art

As noted above, there are many publications linking SCD to various diseases. Thus, the concept of using SCD inhibitors in the treatment of such diseases is well established. When this approach has been shown in several diseases, either by *in vitro* or *in vivo* animal model experiments, the state of the prior art is relatively mature. It is not pure speculation.

By quoting Giutierrez-Juarez, *et al, J. Clin. Invest*, 116:1686-1695, 2006, "If the effects of SCD1 deficiency are confirmed in humans, the pharmacological inhibition of this enzyme should have independent and beneficial effects on both weight gain and insulin action," the Examiner seems to imply that unless human test results are available, the invention is speculative.

However, as noted above, human tests are not required before patent protection can be granted. Again, majority of people skilled in this art believe that inhibition of SCD can be used to treat diseases mediated by SCD. This belief has been substantiated by Sjogren, *et al.* published in 2007 (cited above) and Warensjo, *et al.*, *Obesity* 15:1732-1740, 2007, which also shows a relationship between SCD activity (affected by SCD gene polymorphisms) and obesity/insulin sensitivity in 1143 men.

Applicants respectfully submit that a skilled artisan having knowledge of the above-cited references would not consider treatment of SCD-mediated diseases with SCD inhibitors unpredictable.

5. Working examples

As acknowledged by the Examiner, the specification enables the inhibition of SCD in mice and provides method of assaying SCD inhibition. However, the Examiner argues that the specification does not provide working examples in humans. Again, the legal standard is whether one skilled in the art would believe the animal model correlates with the treatments claimed; there no requirement that a pharmaceutical agent must be demonstrate in human.

It is abundantly clear that one skilled in the art would believe the mouse model would correlate with the human diseases, and this has been demonstrated by Sjogren, *et al.* and Warensjo et al. noted above.

6. Skill of those in the art

Applicant submits that a skilled artisan would be someone with an M.D. and/or Ph.D. degree in the related field.

7. The quantity of experimentation needed

The present Specification provides adequate guidance with a dosage range and methods of administration. Procedures for determining optimal dosage range and optimal method of administration are routine and known to a skilled artisan. The particular area of art is not speculative. Therefore, the Applicant submits that one skilled in the art would not need "undue experimentation" to use the present invention.

Claim Rejections under 35 U.S.C. § 102(a)

Claims 10 and 37 are rejected under 35 U.S.C. § 102(a) as anticipated by Dickson et al. (US 2006009460) (hereinafter "Dickson"), Lu et al. (US 2007203100) (hereinafter "Lu"), Chubb et al., (US 2007185101) (hereinafter "Chubb"), and Dow et al., (US 7247628)

(hereinafter "Dow"), while claims 10, 11, and 37 are rejected under 35 U.S.C. § 102(a) as anticipated by Iwata et al., (JP 2004203871) (hereinafter "Iwata").

The priority dates and publication dates of the above references are as follows:

Dickson: Jun 4, 2004 (publication date: Jan. 12, 2006)

Lu: Feb 24, 2004 (publication date: Aug. 30, 2007)

Chubb: Feb. 14, 2003 (publication date: Aug. 9, 2007)

Iwata: None (publication date: Jul. 22, 2004)

Dow: Dec. 12, 2002 (publication date: Jun. 24, 2004)

Dickson and Lu are not valid prior art references because they were filed after the priority date of the present invention, July 30, 2003. In addition, Iwata is not a valid prior art reference under 35 U.S.C. §102(a) because it was published after the priority date of the present invention, nor is it a prior art reference under 35 U.S.C. § 102(e) because it is not a .U.S. application. Accordingly, withdrawal of rejections based on these references is respectfully requested.

Chubb and Dow were published after the priority date of the present invention. Therefore, they are not prior art under 35 U.S.C. § 102(a). However, they qualify as prior art references under 35 U.S.C. §102(e).

Claim Rejections under 35 U.S.C. § 102(b)

1. Dickson et al.

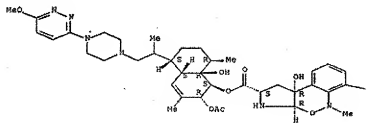
Claims 10 and 37 are rejected under U.S.C. 102(a) as being anticipated by Dickson et al., U.S. 2006009460. As noted above, Dickson et al. is not a proper prior art reference. Accordingly, withdrawal of this rejection is respectfully requested.

2. Lu et al.

Claims 10 and 37 are rejected under U.S.C. 102(a) as being anticipated by Lu et al., U.S. 2007203100. As noted above, Lu et al. is not a proper prior art reference. Accordingly, withdrawal of this rejection is respectfully requested.

3. Chubb et al.

Claims 10 and 37 are rejected under U.S.C. 102(a) as being anticipated by Chubb et al., U.S. 2007185101. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claims, this rejection is respectfully traversed.



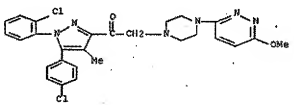
As shown in the above structure, RN 745064-54-4 has a methoxy group attached to the 6-position of the pyridazine ring. This methoxy group correspond to the "R^2-W—" group of compounds of the present invention. However the "R^2-W—" group of compounds of the invention does not include any alkyloxy group. Therefore, compounds of Chubb et al. are structurally distinct from compounds of the invention. In addition, Chubb et al. discloses the use of these compounds as antiparasitic agents, which is different from the use of the compounds of the invention. Accordingly, withdrawal of this rejection is respectfully requested.

4. Iwata et al.

Claims 10, 11, and 37 are rejected under U.S.C. 102(a) as being anticipated by Iwata et al., JP 2004203871. As noted above, Iwata et al. is not a proper prior art reference. Accordingly, withdrawal of this rejection is respectfully requested.

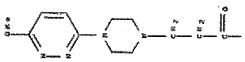
5. Dow et al.

Claims 10 and 37 are rejected under U.S.C. 102(a) as being anticipated by Dow et al., U.S. 7247628. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claims, this rejection is respectfully traversed.



As shown in the above structure, RN 709034-95-7 has a methoxy group attached to the 6-position of the pyridazine ring. This methoxy group correspond to the "R^2-W—" group of compounds of the present invention. However the "R^2-W—" group of compounds of the invention does not include any alkyloxy group. Therefore, compounds of Dow et al. are structurally distinct from compounds of the invention. In addition, Dow et al. discloses the use of these compounds as cannabinoid CB1 receptor antagonists, which is different from the use of the compounds of the invention. Accordingly, withdrawal of this rejection is respectfully requested.

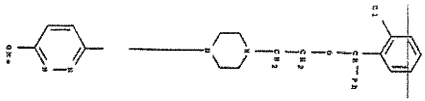
6. CAPLUS AN: 1968:95776



Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Database CAPLUS AN: 1968:95776, which discloses RN 18524-49-7 (2-chloro-10-[3-[4-(6-methoxy-3-pyridazinyl)-1-piperazinyl]propionyl]-phenothiazine). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claims, this rejection is respectfully traversed.

CAPLUS AN: 1968:95776 discloses 2-chloro-10-[3-[4-(6-methoxy-3-pyridazinyl)-1-piperazinyl]propionyl]-phenothiazine. In this compound, the 6-position of pyridazine ring is substituted with methoxy. This methoxy group corresponds to the "R^2-W—" group in compounds of the invention. However, the "R^2-W—" group cannot be any alkyloxy group. Thus, the compound disclosed in CAPLUS AN: 1968:95776 is different from compounds of the invention. Accordingly, withdrawal of this rejection is respectfully requested.

7. CAPLUS AN: 1967:473577



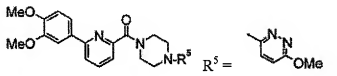
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Database CAPLUS AN: 1967:473577, which discloses RN 15567-65-4 (3-[4-[2-[(o-chloro-α-phenylbenzyl)oxy]ethyl]-1-piperazinyl]-6-methoxy-pyridazine). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

CAPLUS AN: 1967:473577 discloses 3-[4-[2-[(o-chloro-α-phenyl-benzyl)oxy]ethyl]-1-piperazinyl]-6-methoxy-pyridazine. In this compound, the 6-position of pyridazine ring is substituted with methoxy. This methoxy group corresponds to the "R^2-W—" group in compounds of the invention. However, the "R^2-W—" group cannot be any alkyloxy group. Thus, the compound disclosed in CAPLUS AN: 1967:473577 is different from compounds of the invention. Accordingly, withdrawal of this rejection is respectfully requested.

8. Iwata et al. and EP 1396487

Claims 10, 11, and 37 are rejected under 35 U.S.C. §102(b) as being anticipated by Iwata et al. WO 02/102778 (hereinafter "Iwata") (published on Dec. 27, 2002) and EP 1396487 (published on Mar. 10, 2004).

The EP 1396487 application was published after the priority date of the present invention, July 30, 2003. Therefore, it is not a proper prior art reference. Iwata was published on Dec. 27, 2002, which is within 12 months prior to the priority date of the present invention, July 30, 2003. Thus, Iwata is a prior art reference under 35 U.S.C. § 102(a), not 102(b). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



As shown in the above structure, Compound 49 of Iwata has a 6-methoxy-pyridazine group. The methoxy group corresponds to the the "R^2-W—" group in compounds of the invention. However, the "R^2-W—" group cannot be any alkyloxy group. Thus, the compound disclosed in Iwata is different from compounds of the invention. Accordingly, withdrawal of this rejection is respectfully requested.

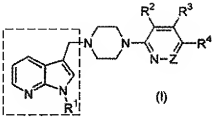
9. Herron et al.

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Herron et al. WO 02/10154 (hereinafter "Herron") (Examples P13-P21, 48, 52, 81, and 85). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

Herron discloses compounds comprising a pyridazine core bonded to a 7-membered homopiperazine core (P13-P21, and Examples 48, 52, 81, and 85). In contrast, the amended claim 10 (x = y = 1) recites compounds comprising a pyridazine core bonded to a 6-membered piperazine core. Therefore, the amended claim 10 is patentable over Herron. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

10. Pollak

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Pollak, et al., WO 99/00386 (hereinafter "Pollak") (Examples 5 and 7). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



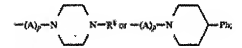
(I)

Pollak discloses compounds having a methyl-1 H-pyrrolo-[2,3-b]pyridine group attached to the N1 position of the piperazine ring, see e.g., 3-14-(6-methoxypyridazin-3-yl)-piperazinyl]methyl-1 H-pyrrolo-[2,3-b]pyridine (Example 5) and 3-[4-(6-methoxy-5-iodopyridazin-3-yl)-piperazinyl]methyl-1 H-pyrrol-[2,3-b]pyridine (Example 7).

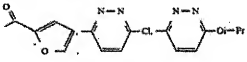
In contrast, compounds of claim 10 have a $-V-R^3$ attached to the N4 position of the piperazine ring. The $-V-R^3$ group does not include methyl-1 H-pyrrolo-[2,3-b]pyridine. Therefore, the amended claim 10 is patentable over Pollak. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

11. Earl

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Earl, et al., US 5166147 (hereinafter "Earl") (Examples 8 and 12). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



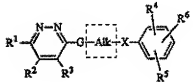
wherein:
(i) A is $-(CH_2)_p-$ (p = 1-5); and/or
(ii) R^3 is selected from the group consisting of:



Earl discloses a compound comprising a piperazine core bonded to a pyridazine core, in which the N4 position of the piperazine is substituted with a $-(CH_2)_p-$ (p = 1-5) linker. This substitution corresponds to the $-V-R^3$ in the compounds of claim 10. However, the $-V-R^3$ group in the compounds of claim 10 cannot have a $-(CH_2)_p-$ directly attached to the N4 of the piperazine ring. Therefore, claim 10 is patentable over Earl and dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

12. Stokbroekx et al. EP 0320032

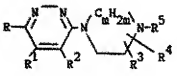
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Stokbrockx, et al., EP 0320032 (hereinafter "Stokbroekx '032") (page 28, Examples 13, 14, and 16; page 33, Example 30; and page 34, Example 88). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Stokbroekx '032 discloses compounds having the above general structure, in which G can be a piperazine and "Alk" is an alkyl linker. The "Alk" group, when attached to N4 of a piperazine ring, corresponds to the $-V-R^3$ group in the compounds of the amended claim 10. However, the $-V-R^3$ group cannot be an alkyl linker directly attached to N4 of the piperazine ring. Therefore, the amended claim 10 is patentable over Stokbroekx '032. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

13. Stokbroekx, et al., EP 0211457

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Stokbroekx, et al., EP 0211457 (hereinafter "Stokbroekx '457") (Examples 6 and 9). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

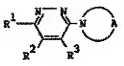


Stokbroekx '457 discloses compounds having the above general structure, in which R5 comprises an alkyl or alkenyl linker directly attached to the N4 of the piperazine ring. In contrast, the corresponding $-V-R^3$ group in the compounds of the amended claim 10 does not have an alkyl or alkenyl linker directly attached to N4 of the piperazine ring. Therefore, the amended claim 10 is patentable over Stokbroekx '457. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

14. Stokbroekx, et al., EP 0156433

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Stokbroekx, et al., EP 0156433 (hereinafter "Stokbroekx '433") (Example 41). Claim 10 has

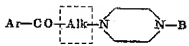
been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Stokbroekx '433 discloses compounds having the above general structure, in which A may comprise a moiety that forms piperazine ring having an R^4 group attached to N4 of piperazine. R^4 is selected from lower alkyl, lower alkylcarbonyl, and lower alkyloxycarbonyl, among others. (p. 3, lines 21-28). The R4 group corresponds to the --V-R^3 group in the compounds of the invention. Claim 10 has been amended such that the –V-R^3 group does not include any of the groups listed in Stokbroekx '433. Therefore, the amended claim 10 is patentable over Stokbroekx '433. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

15. Janssen

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Janssen, US 2985657 (hereinafter "Janssen") (Examples 20 and 24). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Janssen discloses compounds having the above general structure, wherein "Alk" is an alkylene linker directly attached to N4 of the piperazine ring. This "Alk" group corresponds to the –V-R^3 group in the compounds of the invention. Claim 10 has been amended such that the –V-R^3 group does not include an alkylene directly attached to the N4 of the piperazine ring. Therefore, the amended claim 10 is patentable over Janssen. Dependent claim 37 should also be

patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

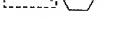
16. Van Emelen et al.

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Van Emelen, et al., WO 03/076422 (hereinafter "Van Emelen") (Examples A21, B17, and B24).

Van Emelen was published on Sept. 18, 2003, which is later than the priority date of the present invention, i.e., July 30, 2003. Therefore, Van Emelen is not a proper prior art reference under 35 U.S.C. § 102(b). Accordingly, withdrawal of this rejection is respectfully requested.

17. Boissier

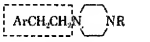
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Boissier, J. Med. Chem. 6:541-544, 1963 (hereinafter "Boissier") (page 542, Example XXIII). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Boissier discloses compounds having the general structure shown above, in which $ArCH_2$- is attached to N4 of the piperazine ring. This "$ArCH_2$-" group corresponds to the –V-R^3 group in the compounds of the invention. Claim 10 has been amended such that the –V-R^3 group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Boissier. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

18. Ratouis

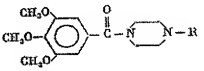
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Ratouis, J. Med. Chem. 8: 104-107, 1965 (hereinafter "Ratouis") (page 105, Example 38). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Ratouis discloses compounds having the general structure shown above, in which $ArCH_2CH_2$- is attached to N4 of the piperazine ring. This "$ArCH_2CH_2$-" group corresponds to the –V-R^3 group in the compounds of the invention. Claim 10 has been amended such that the –V-R^3 group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Ratouis. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

19. Toldy et al.

Claims 10, 11, 15, and 37 are rejected under U.S.C. 102(b) as being anticipated by Toldy et al., Acta Chim. Acad. Sci. Hung., 49:265-286, 1966 (hereinafter "Toldy") (page 268, Example 15). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Toldy discloses compounds having the general structure shown above, in which R may be "6-Methoxypyridazinyl-3-" (compound 15). The 6-Methoxy group on the pyridazine ring corresponds to the R^2-W– group in the compounds of the invention. Claim 10 has been amended such that the R^2-W– group does not include a methoxy group. Therefore, the amended claim 10 is patentable over Toldy. Dependent claims 11, 15, and 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

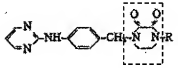
20. Steck et al.

Claim 10 is rejected under U.S.C. 102(b) as being anticipated by Steck et al., J. Heterocycl. Chem. 11:1077-1079, 1974 (hereinafter "Steck") (page 1079). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

Steck discloses various dialkylamino-pyridazines, including 6-(4-Benzyl-1-piperazinyl)-3-diethylaminopyridazine (page 1079, left column). The 4-Benzyl group on the piperazine ring corresponds to the $-V-R^3$ group at the N4 of the piperazine ring in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include any $-CH_2-$ (including the benzyl group; i.e., $Ph-CH_2-$) directly attached to N4 of the piperazine ring. Therefore, the amended claim 10 is patentable over Steck. Accordingly, withdrawal of this rejection is respectfully requested.

21. Hori et al.

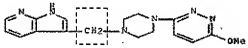
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Hori et al., Chem. Pharm. Bull. 29:1253-1266, 1981 (hereinafter "Hori") (page 1259, Example 17x). Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Hori discloses compounds having the general structure shown above, in which the piperazine ring includes di-oxo substitutions. In contrast, claim 10 has been amended to exclude "oxo" analogs of the piperazine ring. Therefore, the amended claim 10 is patentable over Hori. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

22. Pollak et al.

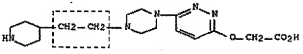
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Pollak et al., US 5976497 (hereinafter "Pollak") describing RN 219635-11-7, RN 219635-16-2, and RN 219635-21-9. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Pollak discloses pyrrolo[2,3-b]pyridine compounds having the general structure shown above, in which the pyrrolo[2,3-b]pyridine moiety is linked via a $-CH_2-$ linkage to the piperazinyl-pyridazine moiety. Thus, the "pyrrolo[2,3-b]pyridinyl-CH_2-" group corresponds to the $-V-R^3$ group at N4 of piperazine in the compounds of the invention. However, the $-V-R^3$ group of the amended claim 10 cannot have a $-CH_2-$ linkage directly attached to N4 of the piperazine ring. Therefore, the amended claim 10 is patentable over Pollak. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

23. Pieper et al.

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Pieper, et al., US 5994356 (hereinafter "Pieper") describing RN 198626-16-3 and RN 198628-02-3. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

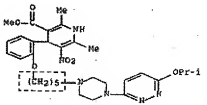


Pieper discloses compounds having the general structure shown above, in which a piperidine is linked via $-CH_2-CH_2-$ to N4 of the piperazine ring. This "piperidinyl-CH_2CH_2-" group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been

amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Pieper. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

24. Earl, et al., US 5166147

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Earl, et al., US 5166147 (hereinafter "Earl") describing RN 146825-54-9. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

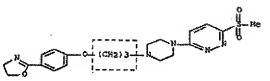


Earl discloses compounds having the general structure shown above, in which an aryloxy group is linked via $-(CH_2)_5-$ to N4 of the piperazine ring. This "aryloxy-$(CH_2)_5$-" group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Earl. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

25. Stokbroekx et al.

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Stokbroekx et al., (hereinafter "Stokbroekx") US 5106973 describing RN 124436-73-3, RN 124436-74-4, RN 124437-24-7, and RN 124437-29-2. Claim 10 has been amended. To the

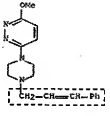
extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Stokbroekx discloses compounds having the general structure shown above, in which an aryloxy group is linked via $-(CH_2)_3-$ to N4 of the piperazine ring. This "aryloxy-$(CH_2)_3$-" group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Stokbroekx. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

26. Janssen, JP 62029575

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Janssen, JP 62029575 (hereinafter "Janssen") describing RN 107746-69-0, RN 107746-75-8, and RN 107746-96-3. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.

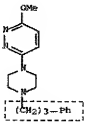


Janssen discloses compounds having the general structure shown above, in which a Ph-CH=CH-CH_2- group is linked directly to N4 of the piperazine ring. This "Ph-CH=CH-CH_2-" group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Janssen. Dependent claim 37

should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

27. Stokbroekx et al., US 5157035

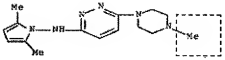
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Stokbroekx et al., US 5157035 (hereinafter "Stokbroekx") describing RN 100241-15-4. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Stokbroekx discloses compounds having the general structure shown above, in which a Ph-$(CH_2)_3$- group is linked directly to N4 of the piperazine ring. This "Ph-$(CH_2)_3$-" group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Stokbroekx. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

28. Bellasio et al.

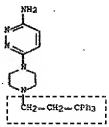
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Bellasio et al., J. Med. Chem. 27:1077-1083, 1984 (hereinafter "Bellasio") describing RN 75841-91-7. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Bellasio discloses compounds having the general structure shown above, in which an alkyl group is linked directly to N4 of the piperazine ring. This alkyl group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkyl or alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Bellasio. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

29. Regnier et al.

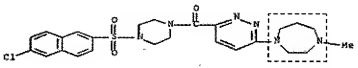
Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Regnier et al., J. Med. Chem. 15:295-301, 1972 (hereinafter "Regnier") describing RN 36524-71-7, RN 36514-72-8, and RN 36524-73-9. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Regnier discloses compounds having the general structure shown above, in which $Ph_3C-CH_2-CH_2-$ group is linked directly to N4 of the piperazine ring. This $Ph_3C-CH_2-CH_2-$ group corresponds to the $-V-R^3$ group in the compounds of the invention. Claim 10 has been amended such that the $-V-R^3$ group does not include an alkylene directly attached to the N4 of piperazine. Therefore, the amended claim 10 is patentable over Regnier. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

30. Herron et al.

Claims 10 and 37 are rejected under U.S.C. 102(b) as being anticipated by Herron et al., WO 2002010154 (hereinafter "Herron") describing RN 395684-35-2 and RN 395685-15-1. Claim 10 has been amended. To the extent that this rejection may still apply to the amended claim, this rejection is respectfully traversed.



Herron discloses compounds having the general structure shown above, in which a seven-membered ring homopiperazine is attached to a pyridazine. Claim 10 has been amended to exclude a seven-membered ring homopiperazine. Therefore, the amended claim 10 is patentable over Herron. Dependent claim 37 should also be patentable for at least the same reasons. Accordingly, withdrawal of this rejection is respectfully requested.

Objected Claims

Claims 12-14 and 16-35 are objected to as being dependent on a rejected base claim. Applicant thanks the Examiner for indicating claims 12-14 and 16-35 contain allowable subject matter. For reasons set forth above, Applicant believes the bases claims, from which these claims depend, are allowable. Therefore, Applicant respectfully defers re-writing these claims in independent form.

Conclusion

Applicant believes this reply is fully responsive to all outstanding issues and places this application in condition for allowance. If this belief is incorrect, or other issues arise, the Examiner is encouraged to contact the undersigned or his associates at the telephone number listed below. Please apply any charges not covered, or any credits, to Deposit Account 50-0591, Reference 17243/004001.

Dated: March 24, 2008

Respectfully submitted,

By: 

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Attachments

(Table 1). Thus, 4 of 6 colorectal tumours had evidence of biallelic inactivation of *MBD4*. We did not test for promoter hypermethylation in the other two tumours, as at present we have not isolated the promoter region of *MBD4*.

The frequency of *MBD4* mutations in HNPCC tumours associated with a known *MSH2/MLH1* germline mutation (6/21, 28.6%) was similar to that in sporadic colorectal tumours lacking *MSH2/MLH1* mutation but displaying loss of MLHI or MSH2 expression by immunohistochemistry (5/21, 23.8%; Table 1). This indicates that *MBD4* is a frequent target of the genomic instability in MSI colorectal carcinomas regardless of their hereditary or sporadic origin. With regard to its proposed role in regulating genomic stability[6], *MBD4* alterations may represent 'mutator's mutations'[7] that contribute to the progressive unfolding of MSI in these tumours, as suggested for *MSH3* and *MSH6* frameshift mutations[2]. Consistent with this, all but two *MBD4* mutations were detected in moderately or poorly differentiated tumours (Table 1).

The identification of additional target genes of genomic instability should clarify the pathogenesis of MSI tumours and possibly improve cancer prevention and therapy[1]. To determine whether an affected gene is a bona fide MSI target, five criteria have been proposed[11]. *MBD4* meets four of these: (i) *MBD4* mutations are frequent; (ii) as a DNA repair gene, *MBD4* would perform a tumour-suppressor function; (ii) functional studies in cell lines support a role for *MBD4* in the maintenance of genomic stability[6]; and (iv) MSI tumours with *MBD4* mutation frequently show biallelic inactivation, either by concurrent alteration of the other allele's coding repeat or by LOH. Overall, these studies further illustrate that in a subset of human carcinomas a mutational process is set in motion that progressively targets DNA repair genes and steadily increases genomic instability.

Acknowledgements

We thank P. Tsichlis and A. Knudson for advice and encouragement; P. Engstrom for support; S. Litwin for statistical analysis; L. Cicchillitti for technical assistance; A. Tosolini for assistance with FISH mapping; and W. Kruger for critical reading of the manuscript. This work was supported by American Cancer Society grant IRG-191, Public Health Service grants CA78412 and CA70328, the Aaron Gold Cancer Prevention Research Fund, the McGorty Foundation, the Millie and Julius Roden Memorial Research Fund, the Italian Association for Cancer Research Special Project 'Hereditary Colorectal Tumors' and Italian National Research Council contract no. 97.04207.CT04. Additional support was provided by Public Health Service grant CA-06927 and by an appropriation from the Commonwealth of Pennsylvania to the Fox Chase Cancer Center.

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Divisions of [1]Population Science and [2]Medical Science, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA. Departments of [3]Medical Genetics and [4]Pathology, University of Helsinki, Helsinki, Finland. [5]Department of Internal Medicine, University of Modena, Modena, Italy. [6]Department of Pathology, Anatomy and Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania, USA. [7]Department of Medical Genetics, Catholic University Medical School, Rome, Italy. Correspondence should be addressed to A.B. (e-mail: A_Bellacosa@fccc.edu).

1. Marra, G. & Boland, C.R. *J. Natl Cancer Inst.* **87**, 1114–1125 (1995).
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Scd1 is expressed in sebaceous glands and is disrupted in the asebia mouse

The sebaceous gland has an important role in hair biology[1]. The asebia (*ab*) mutant mouse has rudimentary sebaceous glands and develops alopecia[2,3]; here we elucidate the genetic basis for this recessive phenotype. Histopathological studies of *ab*[J] (AB/JLe *ab*[J]/*ab*[J]; ref. 3) and recently discovered *ab*[2J] (DBA/1LacJ-*ab*[2J]/*ab*[2J]; J.P.S., unpublished data) allelic mice indicate that the hair shaft in these mice, which is unable to shed its sheath, grows in reverse toward the subcutis, leading to chronic foreign body inflammatory reactions followed by follicle loss and dermal scarring in adult mutant mice. Recently we reported high-resolution genetic mapping of the *ab*[2J] mutation[4]. On the basis of the altered profile of skin surface lipids in mutant mice and the known genes within the 1.6-cM mapped interval, we considered the genes encoding stearoyl-CoA desaturase 1 (*Scd1*) and stearoyl-coA desaturase 2 (*Scd2*) as potential candidates for the *ab* mutation (refs 5–9).

By northern-blot analysis we detected *Scd2* expression in wild-type, *ab*[2J] and *ab*[J] tissues. We did not detect *Scd1* expression in tissues from either *ab*[2J] or *ab*[J] mice (Fig. 1*a*). By RT-PCR we observed a single band of the expected size for *Scd1* in wild-type mice. We detected three bands, possibly representing aberrantly spliced transcripts of 687, 738 and 828 bp, in the *ab*[2J] mutant (Fig. 1*b*). In contrast, *ab*[J] mice lacked *Scd1*-specific bands (Fig. 1*b*). These results suggested normal expression of *Scd2* in *ab* mice, but an absence of *Scd1* expression in *ab*[J] mice and weak, aberrant expression of *Scd1* in *ab*[2J] mice, possibly due to a *Scd1* mutation. We sought direct evidence for this by sequencing RT-PCR products as well as various exons amplified from genomic DNA. We observed a deletion of 18 bp encompassing the exon 2/intron 2-boundary of *Scd1* in *ab*[2J] genomic DNA. As a consequence of the deletion, small amounts of three *Scd1* transcripts containing an in-frame stop codon at the same position in exon 3 arose in *ab*[2J] skin due to aberrant splicing. In contrast to *ab*[2J], Southern-blot data from *ab*[J] mice showed a larger deletion in *Scd1* covering exons 1–4 (Fig. 1*c*). *Scd2*-specific bands remained unaltered in mutant DNA samples (data not shown).

Fluorescent *in situ* hybridization with a *Scd1*-specific RNA probe showed *Scd1* transcripts in the sebaceous gland, but not in the hair follicle or any other cell type in wild-type mouse skin (Fig. 2*a*). Northern-blot data indicated that *Scd1* RNA is regulated throughout the hair cycle in wild-type mice: *Scd1* mRNA levels were very low in one-day neonatal skin (Fig. 2*b*) and in skin containing natural- or induced-cycle[10] telogen hair follicles (Fig. 2*b*,*c*). *Scd1* expression increased in skin containing early anagen follicles from both natural (Fig. 2*b*) and induced hair cycles (Fig. 2*c*). *Scd2* expression also increased (Fig. 2*b*,*c*).

Unlike rodents, which exhibit tissue-specific and inducible expression of *Scd1*

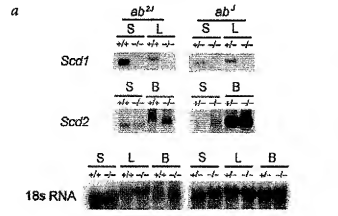

Fig. 1 *Scd* expression and identification of molecular lesions in *ab* mice. **a**, Northern blots of RNA from skin (S), liver (L) and brain (B) probed with *Scd1*- or *Scd2*-specific probes or an ~18S control ribosomal RNA probe. +/+, wild type; -/-, mutant; +/-, heterozygote (wild-type phenotype). **b**, RT-PCR products from ab^J, ab^{2J} and wild-type tissues. Arrowheads indicate *Scd1*- or *Scd2*-specific bands. **c**, Deletion of exons 1–4 in ab^J mouse DNA as seen in Southern blots of *Bam*HI-digested DNA from wild-type and ab^J mice and probed with individual exon-specific probes[5,6], prepared by PCR. Only the *Scd1*-specific bands are indicated.

and *Scd2*, a functional *SCD* and its pseudogene exist in humans[11]. We are unaware of a direct parallel between the *ab* mutation and a human disorder, but the small sebaceous glands and the associated scarring alopecia of mutant mice are reminiscent of some of the clinical scarring alopecias[12].

We conclude that ab^J and ab^{2J} mice lack *Scd1* expression because of their genomic deletions in *Scd1*; hence they can serve as models for *Scd1* deficiency. Specific expression of *Scd1* in wild-type sebaceous glands and the cutaneous pathology seen in the mutant mouse demonstrate the importance of *Scd* product (monounsaturated fatty acids) to normal sebaceous gland function in relation to hair. Although the exact mechanism is unclear at present, fatty acids are known mediators of signal transduction[13,14] and have been implicated in acne[15]. As these mice also suffer from corneal opacities and hypoplastic meibomian glands, the importance of *Scd1* to normal ocular barrier function is also worth noting.

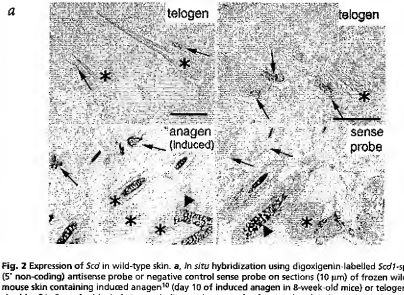

Fig. 2 Expression of *Scd* in wild-type skin. **a**, *In situ* hybridization using digoxigenin-labelled *Scd1*-specific (5′ non-coding) antisense probe or negative control sense probe on sections (10 μm) of frozen wild-type mouse skin containing induced anagen[10] (day 10 of induced anagen in 8-week-old mice) or telogen follicles (day 0 in 8-week-old mice). Arrows indicate sebaceous glands; arrowheads indicate the dark signal due to hair shaft melanin. Small scale bar, 50 μm; large scale bar, 100 μm. **b**, Northern blots of RNA from natural hair cycle dorsal skin of DBA/1LacJ mice at the days (age of mice) indicated and probed with *Scd1* (5′ non-coding), *Scd2* (3′ non-coding) and *Gapd* (control) probes. Day 8 is anagen and days 21 and 42 are telogen. **c**, Northern blots of the induced hair cycle using dorsal skin RNA of C57BL/6 mice. Day 1 is early anagen and days 0 and 20 are telogen follicle skin.

Acknowledgements

We thank M. Eisinger for critical review of the manuscript.

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Fine-mapping of an ancestral recombination breakpoint in *DCP1*

Genetic analysis is necessary to understand the molecular basis of the association between polymorphisms of *DCP1* (encoding the human angiotensin-1 converting enzyme (ACE)), cardiovascular disease and quantitative variation of circulating ACE activity. The recent sequencing of 24 kb of *DCP1* in DNA samples from 6 founders of CEPH families that originate from France and Utah (along with 6 samples from African Americans) revealed 37 polymorphisms[1] that define 3 common haplotypes (H1, H6 and H7) in individuals of European descent. Haplotypes H1 and H7 include the *Alu* deletion allele[2] in intron 16. This work showed "that there is a major genetic subdivision in the deletion clade (H1 and H7) in one of the populations (European-Americans) that enables a more detailed analysis of common cardiovascular traits associated with this polymorphism" (although data for the proposed analysis were not available).

We have previously reported[3], in work cited by Rieder *et al.*[1], an analysis of British *DCP1* haplotypes based on variants discovered by Villard *et al.*[4], and shown how the haplotypes may be grouped into three clades (A, B and C). Clade A is marked by the *Alu* insertion allele and clades B and C by the *Alu* deletion allele (that is, the 'deletion clade' may be subdivided). Moreover, we proposed that clade C was created following an ancestral recombination event between members of clades A and B. We have used the new polymorphism data generated by Rieder *et al.*[1] to refine the location of the putative ancestral breakpoint by genotyping key individuals from our data sets. The results (Fig. 1), combined with our previous data, indicate that the ancestral breakpoint maps downstream of the 6435 polymorphism. Clades A and C may be subdivided on the basis of the 7831 and 8128 polymorphisms, which obviates a more precise localization of the breakpoint with the current data.

We have previously shown in a novel measured haplotype analysis[5] that the major *DCP1*-linked ACE quantitative trait locus is located downstream of the ancestral breakpoint, effectively excluding the *DCP1* promoter. Clades A and B are associated with low and high circulating ACE levels, respectively, and the ACE level associated with the recombinant clade C is similar to that associated with clade B (and statistically indistinguishable in our families; Fig. 2). The refined mapping of the ancestral breakpoint reported here allows us to exclude a further 3.9 kb of *DCP1* including exons 1–5, introns 1–4 and part of intron 5 from harbouring the major ACE-linked quantitative variant. This demonstrates the relevance of the newly discovered variants for the analysis of a common trait associated with the I/D polymorphism. To further refine the localization of the major ACE quantitative variant, *DCP1* haplotypes should be determined in other populations with different recent evolutionary histories to search for additional ancestral breakpoints.

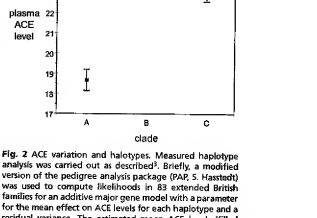

Fig. 2 ACE variation and haplotypes. Measured haplotype analysis was carried out as described[5]. Briefly, a modified version of the pedigree analysis package (PAP, S. Hanstedt) was used to compute likelihoods in 83 extended British families for an additive major gene model with a parameter for the mean effect on ACE levels for each haplotype and a residual variance. The estimated mean ACE levels (filled blocks) associated with each clade are shown together with approximate 95% confidence intervals (error bars).

polymorphism (Rieder *et al.*[1])	(Villard *et al.*[4])	A-1	A-2	B	C-1	C-2
		haplotype				
–2851	T–5491C	T	T	C	T	T
–2826	A–5468C	A	A	C	A	A
–1252	T–3892C	T	T	C	T	T
2400	A–240T	A	A	T	A	A
2547	T–93C	T	T	C	T	T
3872		A	A	G	A	A
4504		G	G	C	G	G
6435		G	G	A	G	G
7831		A	G	G	G	G
8128	T1237C	C	C	T	C	T
8988		C	C	T	T	T
12257	G2215A	G	G	A	A	A
13145		C	C	A	A	A
13230		A	A	G	G	G
13336		A	A	T	T	T
13338		T	T	G	G	G
14094	I/D	I	I	D	D	D
14521	G2350A	A	A	G	G	G
15214		A	A	G	G	G
18222		A	A	G	G	G
20833		C	C	T	T	T
22251		C	C	T	T	T
23945	4656(CT)$_{2/3}$	3	3	2	2	2

Fig. 1 Extended haplotypes for clades A, B and C. Polymorphisms are labelled following the scheme proposed by Rieder *et al.*[1] based on their relative genomic sequence position. The alternative labelling scheme proposed by Villard *et al.*[4] is shown for comparison. Results are shown from 12 individuals homozygous for clades A, B or C selected from British families that have been previously genotyped for 10 *DCP1* polymorphisms[3]. The Rieder *et al.*[1] polymorphisms were typed by standard PCR-based sequencing methods.

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A lipogenic diet in mice with a disruption of the stearoyl-CoA desaturase 1 gene reveals a stringent requirement of endogenous monounsaturated fatty acids for triglyceride synthesis

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Abstract **Stearoyl-CoA desaturase (SCD) catalyzes the de novo biosynthesis of oleate and palmitoleate, which are the major fatty acids found in triglycerides, cholesteryl esters, and phospholipids. A high carbohydrate (lipogenic) diet induces lipogenic gene expression by sterol regulatory element binding protein 1 (SREBP-1c)-mediated gene transcription, leading to an increase in the synthesis of triglycerides. The lipogenic diet fed to mice with a null mutation in the SCD1 gene (SCD−/−) fails to induce the synthesis of triglycerides in liver, despite the induction of expression of SREBP-1 and its target genes, fatty acid synthase and glycerol-3-phosphate acyltransferase. The lipogenic diet led to a decrease in the levels of triglyceride, but an increase in the level of cholesteryl esters of saturated fatty acids. Feeding a lipogenic diet supplemented with high levels of oleate to the SCD−/− mice resulted in incorporation of oleate in the liver of SCD−/− mice, but failed to restore triglycerides to the levels in the normal mouse. Triglyceride synthesis, as measured by the incorporation of [^{3}H]glycerol, was dramatically reduced in the liver of SCD−/− mouse fed a lipogenic diet compared with the normal mouse.** These observations demonstrate that induction of triglyceride synthesis is highly dependent on SCD1 gene expression.—Miyazaki, M., Y-C. Kim, and J. M. Ntambi. **A lipogenic diet in mice with a disruption of the stearoyl-CoA desaturase 1 gene reveals a stringent requirement of endogenous monounsaturated fatty acids for triglyceride synthesis.** *J. Lipid Res.* **2001. 42: 1018–1024.**

Supplementary key words triglycerides • cholesteryl esters • gene expression • SREBP

Stearoyl-CoA desaturase (SCD) is a key enzyme involved in the biosynthesis of unsaturated fatty acids, as well as the regulation of this process. It catalyzes the Δ^9-desaturation of long-chain fatty acids, leading to biosynthesis of palmitoleic (C16:1) and oleic (C18:1) acids as major products (1). Palmitoleic and oleic acids are the major monounsaturated fatty acids of triglycerides, cholesteryl esters, and membrane phospholipids. When fasted animals are subsequently fed a high carbohydrate (lipogenic) diet, there is a dramatic increase in the levels of SCD and several other enzymes involved in fatty acid and triglyceride synthesis such as acetyl-CoA carboxylase, fatty acid synthase (FAS), and glycerol-3-phosphate acyltransferase (GPAT) (2–3). The coordinate induction of these enzymes is due to increased sterol regulatory element binding protein 1 (SREBP-1)-stimulated gene transcription (4–8). The induction of lipogenic gene transcription is followed by a prompt increase in fatty acids and triglycerides to the levels above those observed in animals fed normally (2). The majority of the excess triglycerides are incorporated and secreted by the liver as VLDL-TG for transport to other tissues (9). A high level of VLDL-TG in plasma is a risk factor for cardiovascular disease (10) and, therefore, identifying a control point in the pathway of triglyceride biosynthesis can be a selective target for treating the condition.

Several isoforms of SCD exist in the mouse genome. SCD1 and SCD2, which are products of different genes, are the most well characterized (3, 11, 12). Most organs of different mouse strains express SCD1 and SCD2 with the exception of liver, which expresses mainly the SCD1 isoform. SCD2 is constitutively expressed in the brain (11), and is expressed at high levels in livers of mice that overexpress the truncated nuclear form of SREBP-1a (7). SCD3 is expressed mainly in the skin (12). Several in vivo studies have shown that SCD1 gene expression is highly regulated by dietary changes, hormonal factors, developmental processes, and peroxisomal proliferators (12), implicating the role of SCD in several physiological processes.

Abbreviations: FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; SCD, stearoyl-CoA desaturase; SREBP-1, sterol regulatory element binding protein 1.

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Recent studies using the asebia mutant mouse model have begun to address the role of SCD1 gene expression and its products, the monounsaturated fatty acids, in physiological processes (13–15). The asebia mouse mutation (ab), an autosomal recessive trait characterized by hypoplastic sebaceous glands, arose more than 30 years ago in a colony of BALB/c Crg/Ga mice (15, 16). A similar mutation, designated ab^{j}, arose spontaneously in the BALB/cJ inbred strain at the Jackson Laboratory (Bar Harbor, ME), and was found to be allelic to asebia (16, 17). Using positional and high resolution genetic mapping, the ab^{j} mutation was localized to chromosome 19 and has recently been determined to be in the SCD1 gene (14). Further characterization of the asebia mice showed that they express the SCD2 mRNA isoform in various tissues, but lack the SCD1 gene function (13, 14). In addition to the presence of hypoplastic sebaceous glands, the asebia mice appear hairless with a short sparse hair coat and dry scaly skin (14). The mice also suffer from corneal opacities and hypoplastic meibomian glands, implicating the SCD1 in normal ocular barrier function. In addition, we recently showed that the livers of asebia mice are deficient in hepatic triglycerides and cholesteryl esters (13), suggesting that the SCD1 gene plays an important role in cholesterol and lipoprotein metabolism. The mechanisms leading to these various phenotypes are not yet known, but are postulated to involve deficiencies in the de novo biosynthesis of sufficient monounsaturated fatty acids, mainly oleate and palmitoleate, that serve as the major substrates for the biosynthesis of neutral lipids.

In the present study, we fed a lipogenic diet to mice, and examined the role of SCD1 gene expression in the induction of de novo synthesis of hepatic triglycerides. Normally, a lipogenic diet induces SCD1 gene expression and other lipogenic genes, resulting in increased levels of monounsaturated fatty acids and triglycerides in liver. This feeding regimen allowed us to analyze the fate of endogenously synthesized monounsaturated fatty acids without interference from exogenous sources. We showed that a lipogenic diet failed to induce triglyceride synthesis in a SCD−/− mouse, despite the induction of expression of SREBP-1 and two of its target genes, FAS and GPAT. Feeding a lipogenic diet supplemented with high levels of triolein to the SCD−/− mice resulted in incorporation of oleate into the liver of SCD−/− mice, but failed to restore triglycerides to the levels found in the normal mouse. Further, the rate of triglyceride synthesis, as measured by the incorporation of [^{3}H]glycerol, was dramatically reduced in the livers of SCD−/− mice. Taken together, our results show that the expression of SCD1 is important for the de novo synthesis of monounsaturated fatty acids that act as the main substrates for the dietary induction of triglyceride biosynthesis in liver. Because triglycerides are a major source of stored energy, and most of the hepatic triglycerides are secreted in the form of VLDL for transport to other tissues, the dietary regulation of SCD gene expression may play a larger role in energy and lipoprotein metabolism than was previously thought.

MATERIALS AND METHODS

Animals and diets

Asebia homozygous (SCD−/−) and heterozygous (SCD+/−) mice, originally obtained from the Jackson Laboratory (Bar Harbor, ME), were reared and housed in a pathogen-free animal facility of the department of Biochemistry of the University of Wisconsin–Madison, operating at a 12-h light/12-h dark cycle. The breeding of the animals was in accordance with the protocols approved by the Animal Care Research Committee of the University of Wisconsin–Madison. In this study, the heterozygous mice are considered to have the normal phenotype (13, 14). At 16 weeks of age, the mice were fed, ad libitum for 2 days either chow diet (5008 test diet; PMI Nutrition International Inc., Richmond, IN), high carbohydrate diet (TD 99252, Harlan Teklad, Madison WI), or high carbohydrate diet supplemented with triolein (50% of total fat). The high carbohydrate diet contained, by weight, 21% casein, 14% maltodextrin, 55% sucrose, 5% cellulose, 3% mineral mix (AIN-93G-MX), and 1% vitamin mix (AIN-93-VX).

Materials

Radioactive [^{32}P]dCTP (3,000 Ci/mmol) was obtained from DuPont Co. (Wilmington, DE). TLC plates (TLC Silica Gel G60) were from Merck (Darmstadt, Germany). [1,2,3-^{3}H]-glycerol was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). The cDNA probes for FAS and GPAT were obtained from Dr. H. Sul, University of California, Berkeley. All other chemicals were purchased from Sigma (St. Louis, MO).

Lipid analysis

Total lipids were extracted from liver according to the method of Bligh and Dyer (18), and were separated by silica gel TLC using petroleum ether–diethyl ether–acetic acid (80:30:1) as the developing solvent. The lipids were visualized by cupric sulfate in 8% phosphoric acid. The lipids were scraped, methylated, and analyzed by gas-liquid chromatography as previously described (13). Triheptadecanoylglcerol, heptadecanoyl cholesterol, and 1,2-dihepatadecanoyl-L-α-phosphatidylcholine (Sigma) were added prior to lipid extraction as internal standards for the quantitation of triglycerides, cholesteryl esters, and phospholipids, respectively.

Isolation and analysis of RNA

Total RNA was isolated from livers using the acid guanidinium-phenol-chloroform extraction method (19). Twenty micrograms of total RNA were separated by 1.0% agarose/2.2 M formaldehyde gel electrophoresis, and transferred onto nylon membrane. The membrane was hybridized with ^{32}P-labeled FAS (2), GPAT (20), SREBP-1c (21), and SCD1 cDNA probes (3). pAL15 cDNA probe (19) was used as a control for equal loading of RNA.

In vivo assay for triglyceride synthesis using [^{3}H]glycerol

[1,2,3-^{3}H]glycerol was dissolved in 0.9% NaCl at a concentration of 50 μCi/0.15 ml. Mice that had been fed high carbohydrate diet or chow diet were injected intraperitoneally with 50 μCi of [^{3}H]glycerol 15 min before being sacrificed (22). Hepatic lipids were extracted using Bligh and Dyer's method and separated by TLC using hexane–ether–acetic acid (90:30:1, v/v) as developing solvent. Each lipid extract was scraped off the plate, and the radioactivity was measured using a liquid scintillation counter.

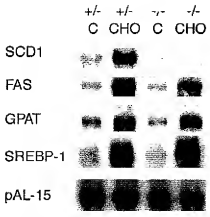

Fig. 1. Northern blot analysis for the expression of SCD1, SREBP-1, FAS, and GPAT mRNA from livers of SCD1−/− and heterozygous (+/−) mice fed a chow (C) or lipogenic diet (CHO). Total RNA (20 μg) pooled from four mice of each group was subjected to Northern analysis followed by hybridization with labeled probes specific for SCD1, SREBP-1, FAS, and GPAT. A cDNA probe for pAL15 (19) was used to confirm equal loading of RNA.

RESULTS

Figure 1 shows a Northern blot of total RNA isolated from the livers of SCD−/− and SCD+/− mice that were fed a chow diet or a lipogenic diet for 2 days and analyzed the expression of SCD1, SREBP-1, FAS, and GPAT mRNAs. There were no noticeable alterations in food intake between the SCD−/− and the wild-type controls. The expression of these genes is induced by a lipogenic diet, resulting in increased levels of fatty acids and triglycerides (2–4). As shown previously (13), the SCD1 mRNA is not detectable in liver of SCD1−/− mice, whereas SCD+/− mice that are phenotypically indistinguishable from normal mice (14) express the 4.9-kb SCD1 mRNA. The SREBP-1, FAS, and GPAT mRNA were detectable in SCD−/− and SCD+/− animals on a chow diet. Upon feeding with a lipogenic diet, the SCD1 mRNA expression was induced 12-fold only in the SCD+/− mouse, whereas the SREBP-1, FAS, and GPAT mRNAs were induced 9.5-, 22-, and 6.5-fold, respectively, in both the SCD−/− and SCD+/− mouse. pAL 15 mRNA expression used as a loading control did not change in both groups of animals on either chow diet or lipogenic diet.

Figure 2 shows TLC analysis of lipids extracted from liver of SCD−/− and SCD+/− mice fed a chow diet or a lipogenic diet. The levels of cholesteryl esters and triglycerides of SCD−/− mice on a chow diet were lower than those of SCD+/− mice, consistent with our previous results (13). When the mice were fed a lipogenic diet, there was a marked increase in triglyceride levels in livers of SCD +/− mice, whereas SCD−/− mice showed a marked reduction in the triglyceride levels. The cholesteryl ester fraction was increased in both SCD+/− and SCD−/− mice. **Table 1** shows that the triglyceride content in liver of SCD+/− mice increased by 2.3-fold, whereas the triglyceride content in the SCD1−/− mice decreased by 59% compared with the animals on a chow diet. The cholesteryl ester fraction was increased by 4.1- and 5.0-fold, respectively, in the livers of the SCD+/− and SCD−/− mice.

Table 2 shows the relative percentage compositions of the major fatty acids measured in the total lipid fraction, as well as in the triglyceride, cholesteryl ester, and phospholipid fractions of livers of SCD+/− and SCD−/− mice fed a lipogenic or chow diet. In the total lipid fraction, the SCD1+/− livers from mice fed a lipogenic diet compared with a chow diet had a 54% increase in the relative amount of 16:1, and a 45% increase in 18:1. In the tri-

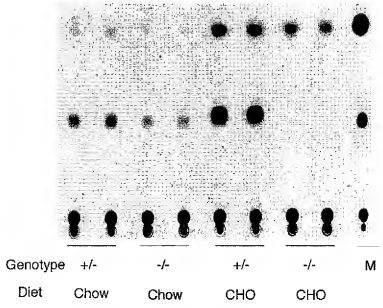

Fig. 2. TLC of lipid extracts from livers of SCD1−/− and heterozygous (SCD+/−) mice fed chow or lipogenic diet for 2 days. Total lipids were extracted from livers of heterozygous and SCD1−/− mice. Lipid extracts were pooled and analyzed by TLC. Equivalent amounts of lipid extract (from 2.5 mg of tissue homogenate) were loaded in each lane. Each lane represents lipids from livers of three mice.

TABLE 1. Phenotypic differences in neu. lipid content between SCD−/− and SCD+/− mice

Lipids	Chow Diet		CHO Diet	
	+/−	−/−	+/−	−/−
	mg/g liver			
Triglycerides	6.0 ± 0.2	3.6 ± 0.7[a]	13.5 ± 1.3[b]	1.5 ± 0.3[c,d]
Cholesteryl esters	1.1 ± 0.0	0.4 ± 0.1[a]	4.5 ± 0.5[b]	2.0 ± 0.4[c,d]
Phospholipids	22.5 ± 0.9	23.3 ± 0.5	19.8 ± 0.4	22.9 ± 1.1

Values are means ± SD (n = 4). Female mice were fed chow diet and lipogenic (CHO) diet for 2 days. Liver triglycerides, cholesteryl esters, and phospholipids were measured as described under Materials and Methods.

[a] $P < 0.01$ between chow diet-fed SCD+/− and chow diet-fed SCD−/− mice.

[b] $P < 0.01$ between chow diet-fed SCD+/− and CHO diet-fed SCD+/− mice.

[c] $P < 0.01$ between chow diet-fed SCD−/− and CHO diet-fed SCD−/− mice.

[d] $P < 0.01$ between CHO diet-fed SCD+/− and CHO diet-fed SCD−/− mice.

glyceride fraction, the relative amount of 16:1 increased by 42%, whereas that of 18:1 increased by 26%. In the liver cholesteryl ester fraction, the relative amount of 16:1 and 18:1 increased by 28% and 15%, respectively. The phospholipid fraction showed a 44% and 38% increase, respectively, in 16:1 and 18:1. In the total lipid fraction, the SCD1−/− livers from mice fed a lipogenic diet compared with the SCD+/− mice had a 100% decrease in the relative amount of 16:1 and a 63% decrease in 18:1. In the hepatic triglyceride fraction, the relative amount of 16:1 decreased by 76%, whereas that of 18:1 decreased by 70%. The liver cholesteryl ester fraction of SCD1−/− mice showed a 67% decrease of 16:1 and a 69% decrease of 18:1, whereas the phospholipid fraction showed a 78% decrease in 16:1 and a 55% decrease in 18:1.

Figure 3 shows the content (mg/g of liver) of the monounsaturated and saturated fatty acids measured in the triglyceride, cholesteryl ester, and phospholipid fractions in the livers of SCD+/− and SCD−/− mice fed a lipogenic diet or chow diet. In the triglyceride fraction of the SCD+/− mouse on a lipogenic diet, the amount of monounsaturated fatty acids increased by 3.1-fold, whereas that of saturated fatty acids increased by 1.9-fold when compared with the chow diet. The liver of SCD−/− mice showed a 97% reduction in the monounsaturated fatty acids compared with the SCD+/− mice. The triglyceride fraction of the SCD1−/− mice showed a 36% decrease in the contents of saturated fatty acids (16:0 and 18:0) compared with the liver of animals on a chow diet. The cholesteryl ester fraction showed a greater than 8.2-fold increase in the content of saturated fatty acids in both the SCD−/− and SCD+/− mice. These data indicate that the increased levels of cholesteryl ester observed in the SCD−/− mouse in response to a lipogenic diet (Fig. 2, Table 1) is most likely due to incorporation of saturated fatty acids, mainly 18:0 and 16:0, in this fraction. The phospholipid fraction also incorporated saturated fatty acids, but to a lesser extent than the cholesteryl ester fraction. Because very low levels of saturated fatty acids were incorporated in the triglyceride fraction, the results would suggest that the triglycerides synthesized in response to lipogenic diet contain mainly monounsaturated fatty acids.

TABLE 2. Fatty acid composition of livers from SCD+/− and SCD−/− mice fed lipogenic diet or chow diet

Lipids	16:0	16:1	18:0	18:1	18:2	20:4	22:6
	%						
Total lipids							
+/−							
Chow	28.5	0.6	17.6	16.2	14.8	14.9	7.4
CHO	28.5	1.5[a]	14.2[a]	29.5[a]	9.6[a]	11.8	5.3
−/−							
Chow	30.3	0.2	20.4	10.3	17.0	13.3	8.5
CHO	31.5[c]	0.0[c]	23.1[b,c]	10.7[c]	14.3[b,c]	12.0	8.4[c]
Triglycerides							
+/−							
Chow	21.2	1.0	5.3	46.2	17.0	2.3	5.9
CHO	18.4[a]	1.7[a]	3.7[a]	62.6[a]	8.5[a]	1.2[a]	3.1
−/−							
Chow	26.9	0.4	10.0	25.8	23.8	2.1	9.9
CHO	29.7[b,c]	0.4[c]	26.6[b,c]	18.7[b,c]	12.5[b,c]	2.6[c]	6.3[c]
Cholesteryl esters							
+/−							
Chow	24.3	4.4	6.6	54.4	8.8	0.8	0.0
CHO	16.9[a]	6.1[a]	5.7	64.3[a]	4.6[a]	2.4[a]	0.0
−/−							
Chow	16.9	3.2	25.4	32.3	18.7	3.5	0.0
CHO	37.4[b,c]	2.0[b,c]	32.4[b,c]	19.8[b,c]	6.1[b]	2.2	0.0
Phospholipids							
+/−							
Chow	22.3	0.5	15.7	11.9	14.4	19.4	13.3
CHO	23.0	0.9[a]	14.4	19.2[a]	10.2[a]	18.9	11.5
−/−							
Chow	23.7	0.1	19.3	6.6	15.7	18.6	14.4
CHO	27.1[b,c]	0.2[c]	18.7[c]	8.6[c]	14.5[c]	15.2	13.8

Liver lipids from individual mice of each group were extracted, and major classes of lipids were separated on TLC. The lipid fractions were methyl-esterified and quantified by gas-liquid chromatography as described under Materials and Methods. Standard errors of the mean were all less than 20% of the mean and are omitted for clarity.

[a] $P < 0.01$ between chow diet-fed SCD+/− and chow diet-fed SCD−/− mice.

[b] $P < 0.01$ between chow diet-fed SCD+/− and CHO diet-fed SCD+/− mice.

[c] $P < 0.01$ between chow diet-fed SCD−/− and CHO diet-fed SCD−/− mice.

To determine whether dietary oleate could substitute for the endogenously synthesized oleate and restore the hepatic triglycerides of the SCD−/− mice to the levels observed in the heterozygous mice, we supplemented the lipogenic diets with high levels of 18:1 (50% of total fat) as triolein, and fed it to the mice for 2 days. There were no noticeable alterations in food intake between the SCD−/− and the wild-type controls. Total liver extracts were prepared, and the lipid fractions were analyzed by TLC. The fatty acid composition of the liver was analyzed by gas-liquid chromatography. **Table 3** shows that feeding diets supplemented with triolein to the SCD−/− mice resulted in an increase in the levels of 18:1 in the liver. However, as shown (**Fig. 4**), there was no recovery in the levels of triglycerides in the SCD−/− mice to the levels observed in the SCD+/− mice fed a lipogenic diet or even to the levels of the SCD−/− mice fed a chow diet. This observation suggests that the increase in the levels of triglycerides in liver in response to a lipogenic diet is largely dependent on the presence of endogenously synthesized oleate.

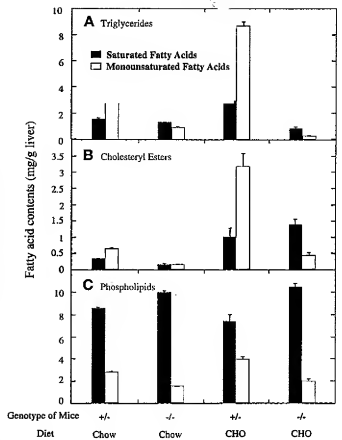

Fig. 3. The contents of saturated and monounsaturated fatty acids in triglyceride, cholesteryl ester, and phospholipid fractions of SCD−/− and SCD+/− mice fed a chow diet or a lipogenic diet. Triheptadecanoylglycerol, heptadecanoyl cholesteryl, and 1,2-dihepatadecanoyl-L-α-phosphatidylcholine were added prior to lipid extraction as internal standards for the quantitation of triglycerides, cholesteryl esters, and phospholipids, respectively. Lipid extracts were pooled and analyzed by TLC, methyl esterified, and quantitated by gas-liquid chromatography.

To establish that the low levels of triglycerides in the SCD−/− mice are due to lower triglyceride synthesis rates, we used [^{3}H]glycerol as a precursor of lipid synthesis to directly measure newly synthesized triglyceride in livers of SCD+/− and SCD−/− mice fed a lipogenic diet. Figure 5 shows that the triglyceride synthetic rate was 7-fold higher in the liver of the heterozygous mice, but only 1.25-fold in the liver of the SCD−/− mice. This figure also shows that although triglyceride synthesis was reduced, that of the phospholipid fraction was increased in both the SCD−/− and SCD+/− mice.

DISCUSSION

It is well known that feeding a lipogenic diet to rodents induces SCD1 gene expression and several other lipogenic genes, leading to high levels of monounsaturated fatty acids and triglycerides (2–4). This feeding regimen allows for the analysis of the fate of de novo synthesized

TABLE 3. F[illegible] acid composition of livers from high oleate diet-fed SCD−/− mice

Lipids	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				%			
Total lipids							
Chow	30.3	0.2	20.4	10.3	17.0	13.3	8.5
CHO	31.5	0.0	23.1	10.7	14.3	12.0	8.4
CHO+18:1	32.3	0.7[a,b]	24.3[c]	24.2[a,b]	6.8[a,b]	6.9[a,b]	4.8[a,b]
Triglycerides							
Chow	26.9	0.4	10.0	25.8	23.8	2.1	9.9
CHO	29.7	0.4	26.6	18.7	12.5	2.6	6.3
CHO+18:1	32.5[c]	0.7[a,b]	26.8[c]	35.4[a,b]	2.5[a,b]	0.7[a,b]	1.0[a,b]

Liver lipids from individual mice of each group were extracted, and major classes of lipids were separated on TLC. The lipid fractions were methyl-esterified and quantified by gas-liquid chromatography as described under Materials and Methods. Standard errors of the mean were all less than 15% of the mean and are omitted for clarity.

[c] $P < 0.01$ between chow diet-fed SCD−/− and high oleate (CHO+18:1) diet-fed SCD−/− mice.

[b] $P < 0.01$ between CHO diet-fed SCD−/− and CHO+18:1 diet-fed SCD−/− mice.

fatty acids without interference from exogenous sources. Using this feeding regimen, we showed that a lipogenic diet fails to induce triglyceride synthesis in SCD−/− mice, despite the induction of expression of SREBP-1, as well as two of its target genes, FAS and GPAT, involved in saturated fatty acid and triglyceride synthesis, respectively. The triglyceride levels, instead, were decreased. We have established that the lower hepatic triglyceride levels are due to a lower rate of triglyceride synthesis in the mutant mice. Because the SCD+/− and the SCD−/− mice consumed equivalent amounts of food, the decrease in the triglyceride levels in the mutant mouse could be due to increased β-oxidation. We also demonstrated that dietary monounsaturated fatty acids could not substitute for the

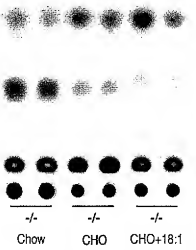

Fig. 4. TLC of lipid extracts from livers of SCD−/− mice fed high oleate (CHO+18:1) diet for 2 days. Total lipids were extracted from livers of SCD−/− mice. Lipid extracts were pooled and analyzed by TLC as described in Materials and Methods. Equivalent amounts of lipid extract (from 2.5 mg of tissue homogenate) were loaded in each lane. Each lane represents lipids from liver of one mouse.

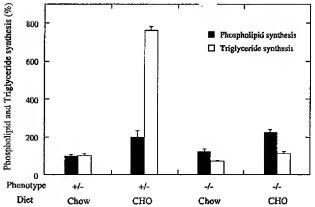

Fig. 5. The rate of glycerolipid synthesis in liver. Mice fed chow diet or lipogenic diet for 2 days were intraperitoneally injected with 50 μCi of [^{3}H]glycerol 15 min before being sacrificed. After extraction of hepatic lipids, phospholipids and triglycerides were separated, and the radioactivity was determined. Data are expressed as a percentage of the values of chow diet-fed heterozygous mice.

ones endogenously synthesized by SCD as substrates for adequate triglyceride synthesis in liver.

The consequences of regulating the SCD1 gene expression by diet may be relevant to lipoprotein metabolism. The majority of de novo synthesis of fatty acids in liver is directed toward triglyceride synthesis and secretion (7). The deficiency in triglyceride levels that we observed could have been due to increased synthesis or secretion of VLDL-TG. However, the lipoprotein profile previously performed on plasma of the SCD−/− showed that these mice have very low levels of triglycerides in the VLDL, LDL, and HDL lipoprotein fractions (13). The current results, therefore, suggest that in the absence of cellular monounsaturated fatty acids due to SCD1 deficiency, the levels of hepatic triglycerides are reduced, leading to lower levels of plasma VLDL-TG.

The use of a lipogenic diet supplemented with high levels of triolein enabled us to test the possibility that the liver could utilize dietary monounsaturated fatty acids instead of those generated in vivo by SCD for de novo synthesis of triglycerides. Dietary oleate or palmitoleate from the small intestine would reach the liver mainly as glycerol esters of chylomicron remnants. The triglycerides are then cleaved into free glycerol and free oleate and palmitoleate in the lysosomes. Because the SCD−/− mice possess normal activity of GPAT (13), this enzyme would then use the freed oleate and palmitoleate as substrates for triglyceride synthesis. We found that feeding a lipogenic diet rich in monounsaturated fatty acids did not correct the deficiency in triglyceride levels in the SCD−/− mice, despite an increase in the levels of 16:1 and 18:1 in the liver. This observation suggests that although the monounsaturated fatty acids reach the liver, they do not necessarily become fatty acyl-CoA substrates at the site of de novo synthesis of liver triglycerides. It is possible that dietary monounsaturated fatty acids and those synthesized endogenously constitute different pools and end up in different environments of the endoplasmic reticulum or cellular compartments where they may be utilized for different purposes. A possible physiological explanation for the requirement of dietary induction of SCD expression is to produce more readily accessible substrates within the vicinity of GPAT to aid in the efficient esterification of glycerol 3-phosphate for triglyceride synthesis.

Feeding a lipogenic diet to the SCD−/− mice led to further accumulation of saturated fatty acids in liver. We found that the saturated fatty acids made endogenously were incorporated into the cholesteryl ester and, to a lesser extent, in the phospholipid fraction, resulting in an increase in the levels of these lipids in liver of both the SCD−/− and SCD+/− mice. However, the saturated fatty acids failed to elevate the levels of the triglyceride fraction in the SCD−/− mice. This observation thus suggests that the endogenously synthesized oleate or palmitoleate, which arise from SCD activity in the endoplasmic reticulum, are critical substrates for the synthesis of triglycerides by GPAT. The results also indicate a more stringent requirement of monounsaturated fatty acids as substrates of diacylglycerol acyltransferase than ACAT. The diacylglycerol acyltransferase step may be important in selecting monounsaturated fatty acids over saturated fatty acids, providing a mechanism to funnel saturated fatty acids into metabolic pools.

The mouse and rat genomes contain several SCD genes, two of which (SCD1 and SCD2) are well characterized (3, 11). These two genes, which are products of two separate genes, are highly homologous at both the nucleotide and amino acid sequence levels, but exhibit divergent tissue-specific expression. Most organs of different mouse strains express SCD1 and SCD2, with the exception of liver, which expresses mainly the SCD1 isoform. In liver, a lipogenic diet and insulin induce SCD1 gene expression, but SCD2 is silent under both conditions (3, 23). The insulin induction of SCD1 gene expression is thought to be mediated by the truncated nuclear form of the SREBP-1c. Liver SCD2 becomes expressed at higher levels only in livers of mice that overexpress SREBP-1 (7). Thus, the transcription of both SCD1 and SCD2 can be regulated by SREBP-1c. We have shown previously that SCD2 mRNA is present in the SCD−/− mice (13), and although expressed at low levels, would be expected to be induced by SREBP-1-mediated gene transcription. However, despite the induction of SREBP-1 by a lipogenic diet and the expression of SCD2 in liver of the SCD−/− mice, the levels of triglycerides remained low, suggesting that SCD2 could not compensate for the SCD1 deficiency. These results point to SCD1 gene expression as a key control point in the induction of triglyceride synthesis in liver.

In conclusion, the present work demonstrates that dietary induction of triglyceride synthesis in mouse liver is highly dependent on the expression of the SCD1 gene. SCD2 cannot compensate for the deficiency. We propose that the synthesis of triglycerides requires endogenously synthesized monounsaturated fatty acids by SCD1 as critical substrates. Dietary monounsaturated fatty acids can get into the liver, but cannot be utilized to synthesize enough triglycerides. Because most of the hepatic triglycerides are

packaged and incorporated into VLDL-TG and transported to tissues such as adipose tissue for storage, the induction of SCD1 gene expression can have a wide range of effects on lipoprotein metabolism in normal and disease states. For instance, high SCD activity has been correlated with high levels of triglycerides in plasma (24). Hypertriglyceridemia is a risk factor for cardiovascular disease, and high SCD expression may be a major cause of this condition in humans. Thus, the regulation of SCD may have broad implications for its potential use as a target in the treatment of human hypertriglyceridemia.

We thank Dr. Alan Attie for useful discussions and critical review of the manuscript. This work was supported, in part, by a grant from the American Heart Association and Xenon Genetics.

Manuscript received 18 January 2001 and in revised form 12 March 2001.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 275, No. 39, Issue of September 29, pp. 30132–30138, 2000
Printed in U.S.A.

The Biosynthesis of Hepatic Cholesterol Esters and Triglycerides Is Impaired in Mice with a Disruption of the Gene for Stearoyl-CoA Desaturase 1*

Received for publication, June 22, 2000, and in revised form, July 13, 2000
Published, JBC Papers in Press, July 17, 2000, DOI 10.1074/jbc.M005488200

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Stearoyl-CoA desaturase (SCD) is a microsomal enzyme required for the biosynthesis of oleate and palmitoleate, which are the major monounsaturated fatty acids of membrane phospholipids, triglycerides, and cholesterol esters. Two well characterized isoforms of SCD, SCD1 and SCD2, exist in the mouse. Most mouse tissues express SCD1 and 2 with the exception of the liver, which expresses mainly the SCD1 isoform. We found that asebia mice homozygous for a natural mutation of the gene for SCD1 (SCD−/−) are deficient in hepatic cholesterol esters and triglycerides despite the presence of normal activities of acyl-CoA:cholesterol acyltransferase and glycerol phosphate acyltransferase, the enzymes responsible for cholesterol ester and triglyceride synthesis, respectively, in the liver of these mice. Feeding diets supplemented with triolein or tripalmitolein to the SCD−/− mice resulted in an increase in the levels of 16:1 and 18:1 in the liver but failed to restore the 18:1 and 16:1 levels of the cholesterol ester and triglycerides to the levels found in normal mice. The SCD−/− mouse had very low levels of triglycerides in the VLDL and LDL lipoprotein fractions compared with the normal animal. Transient transfection of an SCD1 expression vector into Chinese hamster ovary cells resulted in increased SCD activity and esterification of cholesterol to cholesterol esters. Taken together, our observations demonstrate that the oleoyl-CoA and palmitoleyl-CoA produced by SCD1 are necessary to synthesize enough cholesterol esters and triglycerides in the liver and suggest that regulation of SCD1 activity plays an important role in mechanisms of cellular cholesterol homeostasis.

Stearoyl-CoA desaturase (SCD)[1] is a microsomal fatty acid modifying enzyme that catalyzes the introduction of the first double bond between carbons 9 and 10 of saturated fatty acyl-CoA substrates resulting in the production of monounsaturated fatty acids. The preferred substrates for SCD are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (1). Overall, SCD expression affects the fatty acid composition of membrane phospholipids, triglycerides, and cholesterol esters. Effects on composition of phospholipids ultimately determine membrane fluidity, whereas the effects on the composition of cholesterol esters and triglycerides can affect lipoprotein metabolism and adiposity. Thus the regulation of SCD is of considerable physiological importance, and high SCD activity has been implicated in a wide range of disorders including diabetes, atherosclerosis, cancer, and obesity (2).

Two mouse isoforms of SCD, SCD1 and SCD2, which are products of different genes are currently known and are well characterized (2–4). Most organs of different mouse strains express SCD1 and 2 with the exception of liver, which expresses mainly the SCD1 isoform. SCD2 is expressed at higher levels in livers of mice overexpressing the truncated nuclear form of the sterol regulatory element-binding protein-1a (5). Several studies have shown that the SCD1 gene expression is highly regulated by dietary changes, hormonal factors, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (6–8). Oleate, one of the most abundant fatty acids in the diet, is the end product of SCD. Why is it then, that SCD1 is such a highly regulated gene? A clue as to the physiological role of the SCD1 gene and its endogenous products (the monounsaturated fatty acids) has come from recent studies of the asebia (ab) mutant mouse strain (9). The asebia mouse arose from BALB/c mice and was first described by Gates and Karasek (10). They named this mouse strain "asebia" because they found no evidence of sebaceous glands and subsequently reported that the mouse had a mutation that was due to a single autosomal recessive gene with complete penetrance; mice heterozygous for asebia appeared normal (9–11). The recessive mutation was located on chromosome 19 (12) and has recently been determined to be in the SCD1 gene (9). Characterization of the asebia mice (ab/) indicated that the mice express the SCD2 gene isoform in various issues, but SCD1 mRNA expression is not detectable in several mouse tissues so far studied (9). The absence of SCD1 gene expression is due to an extensive deletion in the SCD1 gene in which the first four exons of the gene are missing (9). The asebia mice develop alopecia, reminiscent of some of the clinical scarring alopecias in humans (9). The mice appear hairless with a short, sparse hair coat and dry, slightly scaly skin (10), suggesting the importance of SCD1 expression to the normal development of sebaceous glands and hair growth. In addition, the mice suffer from corneal opacities and hypoplastic meibomian glands, implicating the importance of SCD1 expression in normal ocular barrier function (9). The

* This work was supported by a grant-in-aid from the American Heart Association-Wisconsin affiliate, in part by Grant DAMD17-99-9451 from the Department of Defense (to J. M. N), and by funds from Xenon Genetics Inc. (to M. P. G.-K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SCD, stearoyl-CoA desaturase; ACAT, acyl-CoA:cholesterol acyltransferase; CHO, Chinese hamster ovary cells; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; GPAT, glycerol 3-phosphate acyl transferase; PBS, phosphate-buffered saline.

mechanisms leading to these phenotypes are not known but are postulated to involve deficiencies in the biosynthesis of cellular monounsaturated fatty acids, particularly oleic acid.

In the present study we have examined the role of SCD1 gene expression in the synthesis of hepatic cholesterol esters and triglycerides. The results indicate that SCD1-deficient mice have decreased levels of liver cholesterol esters and triglycerides, despite the presence in liver microsomes of these mice of high activities of ACAT and GPAT, the enzymes that catalyze the synthesis of cholesterol esters and triglycerides, respectively. Additionally, *ad libitum* access of the SCD1-deficient mice to diets rich in oleate and palmitoleate did not restore their hepatic cholesterol esters and triglycerides to the levels found in the normal mice. Upon transient transfection of an expression vector containing the wild type SCD1 cDNA into Chinese hamster ovary (CHO) cells, there was an increase in SCD activity and esterification of cholesterol to cholesterol esters. Taken together, our observations demonstrate that hepatic cholesterol ester and triglyceride synthesis is highly dependent on SCD1 gene expression and suggest that regulation of SCD1 activity plays an important role in mechanisms of cellular cholesterol and lipoprotein homeostasis.

EXPERIMENTAL PROCEDURES

Animals and Diets—Ascbia homozygous (ab^{J}/ab^{J} or −/−) and heterozygous (+/ab^{J} or +/−) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Wisconsin Animal Care Facility. In this study, comparisons are made between the homozygous (−/−) and the heterozygous (+/−) mice because the latter are indistinguishable from normal mice (9, 10). Mice were housed in a pathogen-free barrier facility operating a 12-h light/12-h dark cycle. At 3 weeks of age, these mice were fed *ad libitum* for 2 weeks or 2 months on laboratory chow diet or on a semi-purified diet containing 30% (% of total fatty acids) triolein or tripalmitolein. The semi-purified diet was purchased from Harlan Teklad (Madison, WI) and contained 18% vitamin free casein, 5% soybean oil, 33.55% corn starch, 33.55% sucrose, 5% cellulose, 0.3% L-methionine, 0.1% choline chloride, salt mix (AIN-76A) and vitamin mix (AIN-76A). The fatty acid composition of the experimental diets was determined by gas-liquid chromatography. The control diet contained 11% palmitic acid (16:0), 23% oleic acid (18:1n-9), 53% linoleic acid (18:2n-6), and 8% linolenic acid (18:3n-3). The high triolein diet contained 7% 16:0, 50% 18:1n-9, 35% 18:2n-6, and 5% 18:3n-3. The high tripalmitolein diet contained 6% 16:0, 49% palmitoleic acid (16:1n-7), 12% 18:1n-9, 27% 18:2n-6, and 4% 18:3n-3. Animals were anesthetized at about 10:00 a.m. by intraperitoneal injection of pentobarbital sodium (0.06 mg of Nembutal/g of body weight; Abbot, North Chicago, IL). Liver was isolated immediately, weighed, and kept in liquid nitrogen. Blood samples were obtained from the abdominal vein.

Materials—Radioactive [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from DuPont. Thin layer chromatography plates (TLC Silica Gel G60) were from Merck. [1-^{14}C]Stearoyl-CoA, [^{3}H]cholesterol, and [1-^{14}C] oleoyl-CoA were purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). Immobilon-P transfer membranes were from Millipore (Bedford, MA). ECL Western blot detection kit was from Amersham Pharmacia Biotech, Inc. LT-1 transfection reagent was from PanVera (Madison, WI). All other chemicals were purchased from Sigma. The antibody for rat liver microsome SCD was provided by Dr. Juris Ozols at University of Connecticut Health Center. pcDNA3–1 expression vector SCD1 was provided by Dr. Travis Knight at Iowa State University.

Lipid Analysis—Total lipids were extracted from liver and plasma according to the method of Bligh and Dyer (13), and phospholipids, free cholesterol, triglycerides and cholesterol esters were separated by silica gel TLC. Petroleum ether/diethyl ether/acetic acid (80:30:1) was used as a developing solvent. Spots were visualized by 0.2% 2′,7′-dichlorofluorescein in 95% ethanol or by 10% cupric sulfate in 8% phosphoric acid. The phospholipid, cholesterol ester, and triglyceride spots were scraped, and 1 ml of 5% HCl-methanol was added and heated at 100 °C for 1 h. The methyl esters were analyzed by gas-liquid chromatography using cholesterol heptadecanoate as internal standard (14, 15). Free cholesterol, cholesterol ester, and triglycerides contents of liver and plasma were determined by enzymatic assays (Sigma and Wako Chemicals).

Plasma Lipoprotein Analysis—Mice were fasted a minimum of 4 h and sacrificed by CO_2 asphyxiation and/or cervical dislocation. Blood was collected aseptically by direct cardiac puncture and centrifuged (13,000 × *g*, 5 min, 4 °C) to collect plasma. Lipoproteins were fractionated on a Superose 6HR 10/30 fast protein liquid chromatography column (Amersham Pharmacia Biotech). Plasma samples were diluted 1:1 with PBS, filtered (Cameo 3AS syringe filter, 0.22 μm) and injected onto the column that had been equilibrated with PBS containing 1 mM EDTA and 0.02% NaN_3. The equivalent of 100 μl of plasma was injected onto the column. The flow rate was set constant at 0.3 ml/min. 500-μl fractions were collected and used for total triglyceride measurements (Sigma). Values reported are for total triglyceride mass/fraction. The identities of the lipoproteins have been confirmed by utilizing anti-ApoB immunoreactivity for LDL and anti-Apo A1 immunoreactivity for HDL (not shown).

Isolation and Analysis of RNA—Total RNA was isolated from livers using the acid guanidinium-phenol-chloroform extraction method (16). 20 μg of total RNA was separated by 0.8% agarose/2.2 M formaldehyde gel electrophoresis and transferred onto nylon membrane. The membrane was hybridized with ^{32}P-labeled cDNA probes synthesized using the divergent 5′- or 3′-untranslated regions specific for either the SCD1 (4) or the SCD2 (3) cDNAs. pAL15 probe (16, 17) was used as control for equal loading.

SCD Activity Assay—Stearoyl-CoA desaturase activity was measured in liver microsomes essentially as described by Oshino *et al.* (18). Tissues were homogenized in 10 volumes of buffer A (0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl/1 mM phenylmethylsulfonyl fluoride, pH 7.4). The microsomal membrane fractions (100,000 × *g* pellet) were isolated by sequential centrifugation. Reactions were performed at 37 °C for 5 min with 400 μg of protein homogenate and 27 nM of [1-^{14}C]stearoyl-CoA (60,000 dpm), 1 μM of NADH, 50 μM of Tris/HCl buffer, pH 7.4. After the reaction, fatty acids were extracted and then methylated with 10% acetic chloride/methanol. Saturated fatty acid and monounsaturated fatty acid methyl esters were separated by 10% $AgNO_3$-impregnated TLC using hexane:diethyl ether (9:1) as developing solution. The plates were sprayed with 0.2% 2′,7′-dichlorofluorescein in 95% ethanol, and the lipids were identified under UV light. The fractions were scraped off the plate, and the radioactivity was measured using a liquid scintillation counter (21). The enzyme activity was expressed as nmol min^{-1} mg^{-1} protein

ACAT Activity Assay—ACAT activity was measured essentially as described (19). Reactions were performed at 37 °C for 5 min with 250 μg/ml microsome protein homogenate and 20 μM [1-^{14}C]oleoyl-CoA (18 μCi/μmol). Exogenous cholesterol (20 nmol) was added as complex of Triton WR1339:cholesterol (100:1) to the reaction mixture. Cholesterol esters and triglycerides were separated by TLC using hexane:diethyl ether:acetic acid (90:30:1) as developing solution. The plates were sprayed with 0.2% 2′,7′-dichlorofluorescein in 95% ethanol, and the lipid fractions were identified under UV light. The fractions were scraped off the plate, and the radioactivity was measured with liquid scintillation counter (21).

GPAT Activity Assay—GPAT activity was measured essentially as described by Thomas and Poznansky (25). The incubation mixture contained 75 mM Tris/HCl, pH 7.4, 4 mM $MgCl_2$, 2 mg/ml of bovine serum albumin, 50 μM palmitoyl-CoA, 0.3 mM [^{14}C]glycerol 3-phosphate (7.6 μCi/μmol), and 500 μg/ml microsome protein. The assay mixture was incubated at 37 °C for 10 min, and the reaction was terminated by adding 2 ml of chloroform:methanol (1:2) and 0.6 ml of 1% perchloric acid. After 5 min, the lipids were extracted according to Bligh and Dyer (13). The lower phase was washed three times with 2 ml of 1% perchloric acid. After drying, the radioactivity of lower phase was measured with liquid scintillation counter (21).

Immunoblotting—Pooled liver membranes from five mice of each group were prepared as described by Heinemann and Ozols (20). The same amount of protein (50 μg) from each fraction was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membranes at 4 °C. After blocking with 10% nonfat milk in TBS buffer, pH 8.0, plus Tween at 4 °C overnight, the membrane was washed and incubated with rabbit anti-rat SCD as primary antibody (20) and goat anti- rabbit IgG-horseradish peroxidase conjugate as the secondary antibody. Visualization of the SCD protein was performed with ECL Western blot detection kit.

Transient Transfections of CHO Cell—CHO cells were transfected with pcDNA3–1 mammalian expression vector containing the wild type-mouse SCD1 cDNA (4) or the plant SCD cDNA in the antisense orientation. 10 μg of DNA was transfected with LT-1 transfection reagent into equivalent amounts of cells (1×10^6) plated on 6-cm dishes. Approximately 48 h after transfection, cells were washed twice with PBS, harvested by scraping, and homogenized in 20 mM Tris, 150 mM

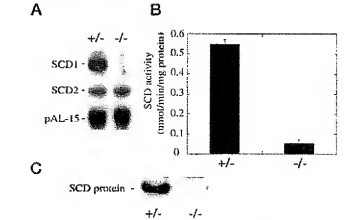

FIG. 1. **Northern blot analysis for the expression of SCD1 and SCD2 mRNA (*A*), SCD enzyme activity (*B*), and immunoblot analysis (*C*) of SCD from livers of SCD1−/− and heterozygous (+/−) mice.** Total RNA (20 mg) pooled from 5 mice of each group was subjected to Northern blot analysis followed by hybridization with labeled probes specific for SCD1 and SCD2 cDNA. A cDNA probe for pAL15 (16) was used to confirm equal loading. For enzyme activity, aliquots of microsome fraction (400 mg) from livers of each group were incubated with a reaction mixture containing [1-^{14}C]stearoyl-CoA for 5 min. The products were saponified and acidified, and the fatty acids were extracted and separated by TLC as described under "Experimental Procedures." Each value represents the mean ± S.D. (n = 5). For immunoblot analysis, aliquots of membrane fraction (80 mg) from pooled livers of each group were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by detection with SCD antibody.

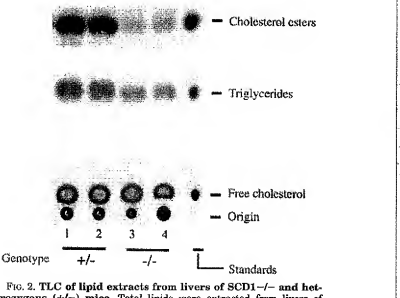

FIG. 2. **TLC of lipid extracts from livers of SCD1−/− and heterozygous (+/−) mice.** Total lipids were extracted from livers of heterozygous and SCD1−/− mice. Lipid extracts were pooled and analyzed by TLC. Equivalent amounts of lipid extract (from 2.5 mg of tissue homogenate) were loaded in each lane. Each lane represents lipids from livers of three mice.

NaCl, and 0.1% Triton X-100 followed by 10 passes through a 25-gauge needle. Total membranes were isolated and used for immunoblotting and for SCD enzyme assay as described above. To measure cholesterol esterification, CHO cultures were incubated with [^{3}H]cholesterol (5 μCi/ml) for 12 h. The cells were washed twice with PBS. Total lipids were then extracted, and the incorporation of [^{3}H]cholesterol into cholesterol esters was measured by TLC followed by liquid scintillation counter (21).

RESULTS

Absence of SCD1 mRNA, Protein, and Enzyme Activity in the Liver of SCD−/− Mice—Northern blot analysis shows that SCD1 mRNA is not detectable in liver of SCD1−/− mice, whereas heterozygous mice (+/−), which are phenotypically indistinguishable from normal mice (9, 10), express the 4.9-kilobase SCD1 mRNA (Fig. 1*A*). SCD2 mRNA was expressed in the livers of both SCD1−/− mice and heterozygous mice at much lower level and was visualized only after a much longer exposure of the autoradiogram. There was no change in the pAL15 mRNA expression, used as a control for equal loading of the RNA. SCD enzyme activity, as measured by the rate of conversion of [1-^{14}C]stearoyl-CoA to [1-^{14}C]oleate (Fig. 1*B*), was high in the heterozygous mice but was decreased by greater than 90% in the total extracts of livers of the SCD1−/− mice. The low level of SCD activity remaining in the extracts of the SCD1−/− mice is probably due to expression of the SCD2 isoform. Immunoblot analysis (Fig. 1*C*) shows that SCD protein was not detectable in the liver microsomes of the SCD1−/− mice, confirming that the antibody is specific for the liver microsomal SCD1 isoform.

SCD1−/− Mice Have Low Levels of Hepatic Cholesterol Esters and Triglycerides of Oleate and Palmitoleate—TLC of lipids extracted from liver of SCD1−/− mice demonstrated markedly reduced cholesterol ester and triglyceride levels compared with the lipids extracted from liver of heterozygous mice (Fig. 2). Table I shows that the total cholesterol ester content in liver of SCD1−/− mice was decreased by 87%. The total plasma cholesterol increased by 35% in the SCD1−/− mice, whereas the liver free cholesterol levels remained the same in both groups of animals. The total liver triglyceride content decreased by 62% in the SCD−/− mice, whereas the plasma triglyceride content decreased by 67%. Plasma lipoproteins were separated by fast performance liquid chromatography, and the distribution of triglycerides among lipoproteins in the various density fractions of the mice are shown in Fig. 3. Lipoprotein profile showed a major difference in the distribution of triglycerides in the VLDL fraction of the SCD−/− and SCD+/− mice. The levels of triglycerides in the SCD+/− were 25 μg/dl in the VLDL, with very low levels in the LDL and HDL fractions. In contrast the SCD−/− had very low levels of triglycerides in the three lipoprotein fractions.

TABLE I
Phenotypic differences between SCD−/− and SCD+/− mice

Female mice were fed control diets for 2 weeks and sacrificed at 5 weeks of age, and liver free cholesterol, cholesterol esters and triglycerides, plasma total cholesterol, and triglycerides were measured. Values are the means ± S.D. (n = 6). Bold values denote a level of statistical significance of $p < 0.001$ between SCD−/− and SCD+/− mice (Student's t test).

Parameter	SCD (+/−)	SCD (−/−)
Body weight (g)	18.9 ± 1.9	18.9 ± 0.7
Liver weight (g)	0.8 ± 0.1	0.9 ± 0.1
Liver free cholesterol (mg/g liver)	1.4 ± 0.1	**1.1 ± 0.1**
Liver cholesterol esters (mg/g liver)	1.5 ± 0.2	**0.2 ± 0.0**
Liver triglycerides (mg/g liver)	9.7 ± 2.3	**3.7 ± 0.3**
Plasma total cholesterol (mg/dl)	49.9 ± 5.9	**76.9 ± 3.0**
Plasma triglycerides (mg/dl)	86.4 ± 2.3	**28.7 ± 2.4**

The major monounsaturated fatty acids of cholesterol esters and triglycerides are palmitoleate and oleate (5). To determine whether the livers of the SCD1−/− mice contained less monounsaturated fatty acids in the cholesterol ester and triglyceride fractions, the lipid fractions were separated by TLC, and the relative fatty acid composition in each fraction was determined. Table II shows the relative percentage of the major fatty acids measured in the cholesterol ester and triglyceride fractions. In the total lipid fraction, the SCD1−/− livers had a 50% decrease in the relative amount of palmitioleate (16:1) and a 43% decrease in oleate (18:1). The relative amount of palmitoleate (16:1) in hepatic cholesterol ester fraction of livers of the SCD1−/− mice decreased by 85%, whereas the relative amount of oleate (18:1) decreased by 60%. Liver triglycerides of

SCD1−/− mice showed an 85% decrease of palmitoleate (16:1) and an 83% decrease of oleate (18:1) compared with the heterozygous mice.

The relative decreases in the monounsaturated fatty acids (16:1 and 18:1) were accompanied by significant increases in the relative percentages of the saturated fatty acids (16:0 and 18:0). Fig. 4 shows the ratio of monounsaturated fatty acids to saturated fatty acids (desaturation index) measured in the cholesterol ester and triglyceride fractions in the livers of the SCD1−/− and heterozygous mice. In the total lipid fraction (Fig. 4, *A* and *D*), the 16:1/16:0 and 18:1/18:0 ratios were decreased in the livers of the SCD1−/− compared with the heterozygous mice. In the cholesterol ester and triglyceride fractions (Fig. 4, *B*, *C*, *E*, and *F*), the ratios of 16:1/16:0 and 18:1/18:0 were decreased by greater than 90%. These data indicate that although the liver cholesterol ester and triglyceride levels are reduced as shown in Fig. 2 and Table I, the absolute monounsaturated fatty acid content in each fraction is dramatically reduced in the SCD1−/− mice with corresponding increases in the saturated fatty acids. There were only minor changes in the composition of other fatty acids. The changes measured in the relative amounts of monounsaturated and saturated fatty acids in the phospholipid fractions were much smaller but followed the trend of the cholesterol esters and triglycerides (data not shown).

Dietary 18:1 or 16:1 Did Not Alter Cholesterol Ester and Triglyceride Levels in the Livers of SCD−/− Mice—The cellular oleate or palmitoleate used for hepatic cholesterol ester and triglyceride synthesis, could be synthesized either *de novo* or by desaturation of exogenous palmitate or stearate indirectly from the diet. To determine whether dietary oleate or palmitoleate could substitute for the endogenously synthesized oleate or palmitoleate and restore the hepatic cholesterol esters and triglycerides of the SCD−/− mice to the levels observed in the heterozygous mice, we supplemented the semipurified mouse diets with high levels of 18:1 or 16:1 (50% of total fat) as triolein or tripalmitolein and then fed these diets to SCD−/− mice for 2 months. Total liver extracts were prepared, and the lipid fractions were analyzed by TLC, and the fatty acid composition of the liver was analyzed by gas-liquid chromatography. Cholesterol esters and triglycerides were also assayed enzymatically and quantitated as mg/g of liver. Feeding diets supplemented with triolein or tripalmitolein to the SCD−/− mice resulted in an increase in the levels of 16:1 and 18:1 in the liver (Table III) but as shown in Fig. 5*A*, there was no recovery in the synthesis of cholesterol esters and triglycerides in the SCD−/− mice to the levels observed in the heterozygous mice. Instead, as shown in Fig. 5*B*, there was an 85 and 60% reduction in the cholesterol esters and triglycerides, respectively, in the livers of SCD−/− mice fed with high 18:1 or 16:1. Taken together, these observations suggest that the synthesis of cholesterol esters and triglycerides in liver is dependent on the presence of endogenously synthesized palmitoleate and oleate.

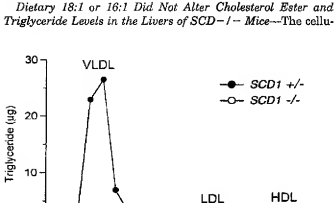

FIG. 3. **VLDL-triglyceride levels in SCD1−/− and SCD1+/− mice.** Plasma lipoproteins were separated by fast performance liquid chromatography, and the distribution of triglycerides among lipoproteins in the various density fractions of the mice was measured. ○, SCD−/−; ●, SCD1+/−. The lipoprotein peaks for VLDL, LDL, and HDL are indicated.

Reduced Levels of Hepatic Cholesterol Esters and Triglycerides in the SCD1−/− Mice Is Not Due to Lack of ACAT or GPAT—The low levels of cholesterol esters and triglycerides observed in the SCD−/− mice could have resulted from reduced levels of ACAT or GPAT, the enzymes that catalyze the synthesis of cholesterol esters and triglycerides, respectively. ACAT activity as measured by the rate of conversion of [1-^{14}C]oleate to labeled cholesterol oleate (Fig. 6) was present at almost equal levels in the liver microsomes of both SCD−/− and heterozygous mice. Cholesterol as a substrate of esterification was not limiting because the free cholesterol levels in the liver of the SCD−/− and heterozygous mice were similar (Table I). GPAT activity, as measured by the esterification of palmitoyl-CoA to radioactive glycerol 3-phosphate, was also similar in both groups of animals (Fig. 6). These observations further confirm the requirement of endogenously synthesized oleate or palmitoleate as the limiting substrates for cholesterol ester and triglyceride synthesis in liver.

Cholesterol Esterification Is Enhanced by SCD1 Gene Overexpression—To confirm that the synthesis of cholesterol esters is dependent on the expression of the SCD1 gene, we transiently expressed wild type mouse SCD1 cDNA (4) in cultured CHO cells and measured cholesterol esterification by labeling

TABLE II

Fatty acid composition of livers of SCD −/− and SCD +/− mice

Female mice were fed control diets for 2 weeks and sacrificed at 5 weeks of age. Liver samples from each mouse were extracted, and the major classes of lipids were separated by TLC. The lipid fractions were methyl-esterified and quantitated by gas-liquid chromatography as described under "Experimental Procedures." Only the major fatty acids are presented. Bold values denote a level of statistical significance of $p < 0.05$ between SCD−/− and SCD+/− (Student's *t* test).

Genotype of mice	Fatty acid composition								
	16:0	16:1n-7	18:0	18:1n-9	18:2n-6	18:3n-3	20:3n-6	20:4n-6	22:6n-3
	% *of total*								
Total fatty acids									
+/−	26.8	**1.0**	**14.7**	14.1	22.0	**0**	2.5	16.4	**0.1**
−/−	27.1	**0.5**	**21.8**	8.1	20.8	**0**	2.2	17.8	**0.1**
Cholesterol esters									
+/−	**23.4**	**2.7**	**5.6**	**34.4**	**25.3**	**1.1**	**1.3**	2.7	**0.5**
−/−	**29.0**	**0.4**	**17.6**	**13.9**	29.6	**1.3**	1.4	3.0	**1.1**
Triglycerides									
+/−	**23.5**	**3.4**	**14.3**	**40.7**	**13.7**	**0.8**	0	**1.4**	0
−/−	**33.8**	**0.5**	**38.5**	**7.1**	**13.9**	**0.9**	0	**5.0**	0

the cultured cells with [^{3}H]cholesterol for 12 h, followed by TLC analysis of the lipid fraction and scintillation counting (21). The transfection of the SCD1 cDNA resulted in a 3–4 fold increase in SCD enzyme activity (Fig. 7*A*) and 5–7-fold increase in protein level (Fig. 7*B*) compared with the control cells that had been transfected with the plant SCD in the antisense orientation. The increase in SCD enzyme activity in the SCD1-transfected cells resulted in increased esterification of cholesterol to cholesterol esters at levels 2-fold higher than in control cells (Fig. 7*C*). These *in vitro* results suggest that during the synthesis of cholesterol esters, the majority of cellular palmitoleate and oleate were required, because substrates of esterification must be made *de novo* by the SCD1 gene.

DISCUSSION

Stearoyl-CoA desaturase has been implicated as a regulatory enzyme in lipogenesis, because stearoyl-CoA desaturation is the rate-limiting step in the overall *de novo* synthesis of unsaturated fatty acids from acetyl-CoA in animal tissues (7). Why SCD is highly regulated and yet oleate, the major product of this enzyme is one of the most abundant fatty acid in the diet has not been well addressed. For instance, dietary oleate is well known for its hypotriglyceridemic effects and yet high SCD activity has been implicated in diabetes, obesity, and atherosclerosis in several animal models (7). By using the asebia mutant mouse strain that has a null mutation in the SCD1 gene as a model, we have begun to address the physiological role of SCD gene expression in normal and disease states. We found that the asebia mice have a deficiency in cholesterol esters and triglycerides in liver and demonstrated by expression of the SCD1 gene in CHO cells that the synthesis of cholesterol esters, is dependent on SCD1 gene expression. This observation suggests that endogenously synthesized monounsaturated fatty acids most likely serve as the main substrates for the synthesis of cholesterol esters. Our observations also indicate that endogenously synthesized monounsaturated fatty acids most likely serve as the main substrates for the synthesis of hepatic triglycerides. The alteration in the levels of monounsaturated fatty acids as a result of SCD1 gene expression can therefore have a wide range of effects on cholesterol and lipoprotein metabolism.

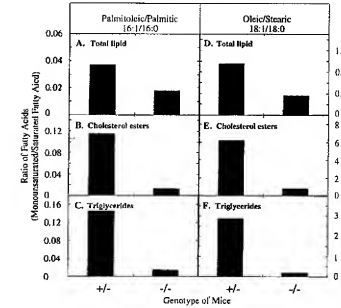

FIG. 4. **The ratio of palmitoleate (16:1) to palmitate (16:0) and oleate (18:1) to stearate in cholesterol ester and triglyceride fraction of SCD−/− and heterozygous (+/−) mice.** Total lipids were extracted from livers of SCD−/− and heterozygous mice. Lipid extracts were pooled and analyzed by TLC, methyl esterified, and quantitated by gas-liquid chromatography. The values used for calculating the ratio of monounsaturated to saturated fatty acids are derived directly from the values presented in Table III.

It has always been perceived that dietary intake is ultimately the major lipid source of monounsaturated fatty acids that get incorporated in triglycerides and cholesterol esters. The concept that SCD modulation in animal tissues differs from feeding animals diets rich in oleate or monounsaturated fatty acids has not been rigorously tested. Dietary oleate or palmitoleate from the small intestine would reach the liver mainly as cholesterol esters of chylomicron remnants. The cholesterol esters are then cleaved into free cholesterol and free oleate and palmitoleate in the lysosomes. ACAT or GPAT would then use the free oleate and palmitoleate as substrates for cholesterol ester or triglyceride synthesis. We tested the possibility that dietary oleate and palmitoleate would serve as substrates of ACAT or GPAT and correct the deficiency in cholesterol esters and triglycerides in the SCD1−/− mice by supplementing the semipurified diet with high levels of oleate or palmitoleate. After several weeks of feeding, our results indicated that dietary oleate or palmitoleate could not correct the deficiency in cholesterol ester and triglyceride levels in the SCD−/− mice despite an increase in the levels of 16:1 and 18:1 in the liver as well as normal activities of ACAT and GPAT. This observation may be explained if we consider that dietary oleate or palmitoleate does not necessarily increase the availability of nonesterified oleate or palmitoleate at the very site of cholesterol ester or triglyceride synthesis. The endogenously synthesized oleate or palmitoleate, which arise from SCD activity in the endoplasmic reticulum, would be better substrates for ACAT and GPAT. When we expressed the mouse SCD1

TABLE III

Fatty acid composition of liver from mice subjected to various dietary paradigms

Male mice were fed control, high 18:1, or high 16:1 diets for 2 months and sacrificed at 13 weeks of age. Liver samples from each mouse were extracted, and the major classes of lipids were separated by TLC. The lipid fraction were methyl esterified and quantitated by GLC as described under "Experimental Procedures." Only the major fatty acids are presented.

Genotype of mice dist		16:0	16:1	18:0	18:1n-9	18:2n-6	18:3n-3	20:4n-6	22:6n-3
Total fatty acids									
+/−	Control	26.6	1.1	16.4	21.1	15.5	0.3	16.7	0.6
−/−	Control	31.4[a]	0.2[a]	23.6[a]	5.6[a]	19.9[a]	0.3	17.6	0.8
−/−	18:1	31.6[a]	0.1[a]	22.1[a]	13.3[a,b]	16.8	0.2	14.2	1.3[a,b]
−/−	16:1	33.7[a]	1.4[b]	25.6[a]	5.9[a]	16.9	0.3	12.9[b]	1.2[a,b]
Cholesterol esters									
+/−	Control	21.1	3.1	10.3	45.2	17.2	0.7	0.9	0.4
−/−	Control	33.2[a]	0.5[a]	31.7[a]	10.3[a]	20.3[a]	0.9	1.8[a]	1.0[a]
−/−	18:1	34.8[a]	0.4[a]	28.9[a]	12.3[a]	17.7	0.9	1.9[a]	1.1[a]
−/−	16:1	35.1[a]	0.7[a]	32.1[a]	10.2[a]	16.4	1.1[a]	1.9[a]	1.5[b]

[a] $p < 0.01$ *vs.* heterozygous mice fed control diet.
[b] $p < 0.01$ *vs.* homozygous mice fed control diet.

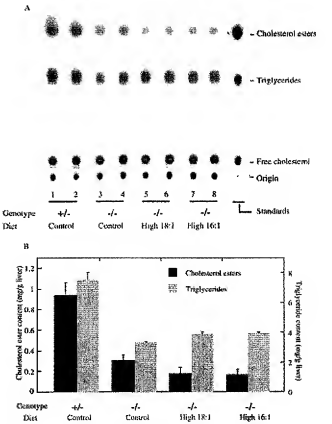

FIG. 5. **TLC of lipid extracts from livers of SCD−/− and heterozygous mice fed high oleate and palmitoleate diets for 2 months.** *A*, total lipids were extracted from livers of SCD−/− and heterozygous mice. Lipid extracts were pooled and analyzed by TLC as described under "Experimental Procedures." Equivalent amounts of lipid extract (from 2.5 mg of tissue homogenate) were loaded in each lane. Each lane represents lipids from liver of one mouse. *B*, total lipids were extracted from 5 mg of liver tissue and separated by TLC using hexane:diethyl ether:acetic acid (90:30:1). The cholesterol ester and triglyceride contents were determined by enzymatic assays. Each value represents the mean ± S.D. ($n = 3$).

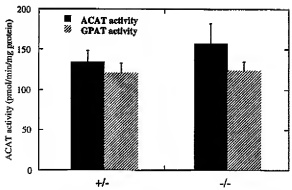

FIG. 6. **ACAT and GPAT activities in liver microsomes of SCD−/− and heterozygous mice.** For ACAT assay, microsomal protein (250 μg) was incubated with a reaction mixture containing [1-^{14}C]oleoyl-CoA for 5 min. The products were saponified and acidified, and the fatty acids were extracted and separated by TLC as described under "Experimental Procedures." GPAT assay was performed at 37 °C for 10 min without exogenous cholesterol. GPAT activity was measured by incubating 500 μg/ml microsome protein with a reaction mixture containing [^{14}C]glycerol 3-phosphate. The lipids were extracted, and the radioactivity of the synthesized triglyceride was measured as described under "Experimental Procedures." Each value for the ACAT or GPAT assay represents the mean ± S.D. ($n = 4$).

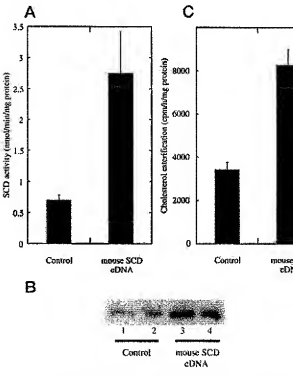

FIG. 7. **Cholesterol esterification activity in Chinese hamster ovary cells transfected with the mouse SCD1 cDNA.** CHO cells were transiently transfected with plasmid cDNA vectors containing wild type mouse SCD1 cDNA or the plant SCD cDNA vector in the antisense orientation as a control. Cells were harvested and homogenized, and total membranes were isolated. SCD activity and protein were analyzed as described under "Experimental Procedures." For SCD activity (*A*), the data are shown for four samples of a single transfection experiment. Each lane in *B* represents protein sample from a single transfection experiment. For cholesterol esterification (*C*), CHO cultures were incubated with [^{3}H]cholesterol (5 μCi/well) for 12 h. The cells were washed twice with PBS. Then, total lipids were extracted from cells as described under "Experimental Procedures." The incorporation of [^{3}H]cholesterol into cholesterol esters was measured by liquid scintillation counter.

cDNA in CHO cells there was increased expression of SCD protein and enzyme activity with a corresponding increase in esterification of cholesterol to cholesterol esters. Although this could be artifact of overexpression of the SCD1 cDNA, this *in vitro* result demonstrates that SCD1 gene expression is required to generate monounsaturated fatty acids as the preferred substrates for cholesterol ester synthesis. Because SCD, ACAT, and GPAT are endoplasmic reticulum membrane enzymes, a possible physiological explanation for the requirement of SCD expression is to produce more readily accessible substrate within the vicinity of ACAT and GPAT to aid in the efficient esterification of cholesterol and glycerol 3-phophate for cholesterol ester and triglyceride synthesis, respectively.

The mouse and rat genomes contain two well characterized structural genes (SCD1 and SCD2) that are highly homologous at the nucleotide and amino acid level and encode the same functional protein (3, 4). The two SCD genes are products of different genes, but despite the fact that both genes are structurally similar, sharing ~87% nucleotide sequence identity in the coding regions, their 5′-flanking regions differ, resulting in divergent tissue-specific gene expression. As shown in the present investigation, despite the expression of the SCD2 in liver of SCD1−/− mice, the full esterification of cholesterol could not occur, suggesting that the SCD2 isoform could not compensate for the SCD1 deficiency. The physiological significance of having two or more mouse SCD isoforms expressed in the same tissue is not currently known but could be related to the substrate specificity of each SCD isoform or the means by which cells compartmentalize lipid biosynthesis for specific functions.

The absence of SCD protein in liver microsomes (Fig. 1C) but residual activity in the total extracts may suggest that SCD1 and SCD2 reside in different cellular compartments.

The consequences of the changes in expression of SCD are relevant to cholesterol and lipoprotein homeostasis. The majority of endogenous synthesis of fatty acids in liver is directed toward cholesterol ester and triglyceride synthesis and secretion (5). Oleic acid, the major product of SCD, is the preferred substrate for ACAT, the enzyme responsible for esterification of cholesterol (22). SCD activity was shown to increase significantly in liver of rats fed high cholesterol diets with a corresponding increase in cholesterol oleate content (23). Additionally, overexpression of sterol regulatory element-binding proteins-1a, 1c, and 2 in mouse liver has been shown to induce SCD1 mRNA and enzyme activity, resulting in the dramatic increase in cholesterol esters and triglycerides of oleate and palmiteolate (5). The increased esterification of cholesterol would prevent the toxic accumulation of free cholesterol in liver, and the increase in the availability of cholesterol esters and triglycerides would lead to their secretion by the liver in the form of VLDL (24). We observed a dramatic decrease in total cholesterol esters and triglycerides in liver and plasma of SCD −/− mice (Table I). Very low levels of VLDL particles with very low levels of triglycerides were present in the SCD−/− mice, suggesting that SCD1 deficiency affects VLDL synthesis, secretion, or possibly clearance from plasma.

In conclusion, the present work demonstrates that cholesterol ester and triglyceride synthesis in mouse liver is highly dependent on the expression of the SCD1 gene. We propose that dietary oleate or palmiteolate does not necessarily increase the availability of nonesterified oleate or palmitoleate at the very site of cholesterol ester and triglyceride synthesis in liver. Normally cholesterol esters are stored in liver hepatocytes or together with triglycerides can be packaged into VLDL and secreted in plasma for transport to other tissues. However, in the absence of cellular monounsaturated fatty acids because of SCD1 deficiency, the levels of hepatic cholesterol esters and triglycerides are reduced, leading to changes in cellular cholesterol levels and plasma lipoprotein profile. Thus, the SCD1 gene may be another checkpoint in the process of cholesterol homeostasis and lipoprotein metabolism and may have broad implications for its potential use as a target in human disease.

Acknowledgments—We thank Dr. Juris Ozols (University of Connecticut Health Center, Farmington, CT) for the antibody to the rat liver microsome stearoyl-CoA desaturase and Dr. Travis Knight (Iowa State University) for the construction of the pcDNA3–1 expression vector for the mouse stearoyl-CoA desaturase. We thank Dr. Sundberg (Jackson Laboratory) and Drs. Stenn, Parimoo, and Prouty (Johnson and Johnson) for useful discussions. We thank Henry Bene for critical review of this manuscript.

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Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol

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Abstract The lipid composition of cellular membranes is regulated to maintain membrane fluidity. A key enzyme involved in this process is the membrane-bound stearoyl-CoA desaturase (SCD) which is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids. A proper ratio of saturated to monounsaturated fatty acids contributes to membrane fluidity. Alterations in this ratio have been implicated in various disease states including cardiovascular disease, obesity, non-insulin-dependent diabetes mellitus, hypertension, neurological diseases, immune disorders, and cancer. The regulation of stearoyl-CoA desaturase is therefore of considerable physiological importance and its activity is sensitive to dietary changes, hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds. Two mouse and rat SCD genes (SCD1 and SCD2) and a single human SCD gene have been cloned and characterized. In the past several years we have studied the dietary influences on the genetic expression of the mouse stearoyl-CoA desaturase. The expression of the mouse SCD genes is regulated by polyunsaturated fatty acids and cholesterol at the levels of transcription and mRNA stability. Promoter elements that are responsible for the polyunsaturated fatty acid repression colocallize with the promoter elements for SREBP-mediated regulation of the SCD genes. It is the goal of this review to provide an overview of the genetic regulation of the stearoyl-CoA desaturase in response to dietary polyunsaturated fatty acids and cholesterol.—Ntambi, J. M. **Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol.** *J. Lipid Res.* 1999. 40: 1549–1558.

Supplementary key words stearoyl CoA desaturase • polyunsaturated fatty acids • cholesterol • lipid metabolism • SREBPs • gene expression

STEAROYL-CoA DESATURASE: WHAT DOES IT DO?

It has been recognized that irrespective of diet, the major storage fatty acids in human adipose tissue are oleate and palmitoleate (1). Why this is the case has not been satisfactorily addressed. There appears to be a number of answers at different levels, i.e., special physico-chemical properties of oleic acid at body temperature, its effects on membrane fluidity, a readily available energy source, and the constraints of the intermediary metabolism of carbohydrates and fats. During the de novo synthesis of fatty acids, the fatty acid synthase enzyme stops at palmitate but the end product of the pathway is usually oleic acid. Stearate is too insoluble to be stored (2). High carbohydrate feeding also leads to high levels of palmitoleate as seen in neonates (2). Adipose fatty acid composition of monounsaturated fatty acids is considered to reflect dietary intake (3) but it is not an exact mirror suggesting the requirement of de novo biosynthesis of the monounsaturated fatty acids in addition to the dietary sources.

A critical committed step in the biosynthesis of monounsaturated fatty acids is the introduction of the first *cis*-double bond in the Δ9 position (between carbons 9 and 10). This oxidative reaction is catalyzed by the iron-containing, microsomal enzyme stearoyl-CoA desaturase (SCD) and involves cytochrome b_5, NADH (P)-cytochrome b_5 reductase, and molecular oxygen (Fig. 1). Although the insertion of a double bond occurs in a spectrum of methylene-interrupted fatty acyl-CoA substrates including *trans*-11 octadecenoic acid (4), the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted into palmitoleoyl- and oleoyl-CoA, respectively (5–8). Overall, Δ9 desaturase affects the fatty acid composition of phospholipids and triglycerides. Effects on phospholipid composition are important in the maintenance of membrane fluidity and alterations have been implicated in a variety of disease states (6). Two mouse and rat genes (SCD1 and SCD2) and a single human SCD gene have been cloned and characterized (9–12). Other SCD cDNAs and genes have been isolated from different species including yeast (13), ovine (14), and hamster (15), and the regulation of their expression is currently being studied by several research groups. The two mouse genes have been shown to

Abbreviations: SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element binding protein; C/EBP, CCAAT enhancer binding protein; PPRE, peroxisome proliferator response element; PUFA-RE, polyunsaturated fatty acid response element; PUFA-BP, polyunsaturated fatty acid binding protein; VLDL, very low density lipoprotein; LXRα, nuclear oxysterol receptor; RXR, retinoic X receptor.

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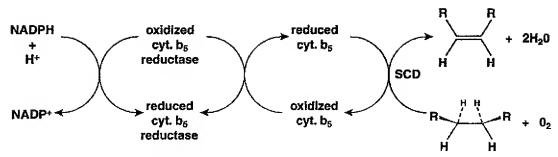

Fig. 1. The pathway of electron transfer in the desaturation of fatty acids by stearoyl-CoA desaturase.

be regulated differently in different tissues, presenting a unique model to study tissue-specific gene expression. The tissue distribution and the dietary alteration of the mouse and rat SCD1 mRNA differ markedly from that of SCD2, being constitutive in adipose tissue, and are markedly induced in liver in response to feeding a high carbohydrate diet (9, 10, 16). Like SCD2, SCD1 mRNA is expressed to a lesser extent in kidney, spleen, heart, and lung in response to a high carbohydrate diet. The SCD2 expression in brain is induced during the development of neonatal mice (17) and down-regulated during the development of mouse lymphocytes (18). The reason for having two SCD isoforms is not known but could be related to the substrate specificities of the two isomers and their regulation through tissue-specific expression. Although certain regions of the promoter of the two mouse genes differ markedly, there is one region with high nucleotide sequence homology. The sequence between −201 to −54 in the SCD2 gene is 77% identical to the sequence between −472 to −325 in the SCD1 gene (Fig. 2). The regulatory elements responsible for polyunsaturated fatty acid and cholesterol regulation of the SCD genes are contained in the sequences within the conserved region. In the past several years we have studied the genetic regulation of the mouse stearoyl-CoA desaturase by dietary and hormonal factors and also during fat cell differentiation. This review will focus on our current understanding of the genetic regulation of the stearoyl-CoA desaturase in response to dietary polyunsaturated fatty acids and cholesterol.

INFLUENCE OF DIETARY FAT ON STEAROYL-CoA DESATURASE IN DISEASE STATES

Oleic acid and palmitoleic acid are the major monounsaturated fatty acids in fat depots and membrane phospholipids. These fatty acids are synthesized by the stearoyl-CoA desaturase. The ratio of stearic acid to oleic acid is one of the factors influencing membrane fluidity and cell–cell interaction (6). Abnormal alteration of this ratio has been shown to play a role in several physiological and disease states including diabetes, cardiovascular disease, obesity, hypertension, neurological diseases, immune disorders, cancer, and aging (19–36).

SCD was viewed as a lipogenic enzyme not only for its key role in the biosynthesis of monounsaturated fatty acids but also for its pattern of regulation by diet and insulin. SCD activity was decreased in rat liver during starvation and diabetes and was rapidly induced to high levels upon refeeding high carbohydrate diets or upon insulin

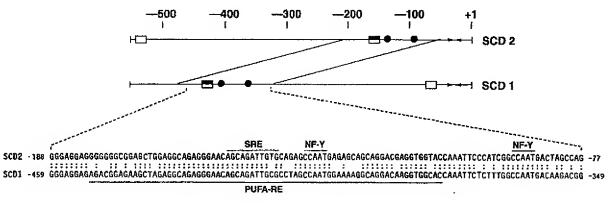

Fig. 2. Organization of the mouse SCD1 and SCD2 promoters and the nucleotide sequence similarity of the 110-bp segment in the 5′-flanking region of the mouse SCD2 and SCD1 genes. SRE, sterol regulatory element; NF-Y, CCAAT-binding factor or nuclear factor Y binding site; C/EBP, CCAAT enhancer binding protein sequence.

administration (37–39). Diets rich in saturated fatty acids or cholesterol also induced desaturase activity in rat liver (40–42). On the other hand, dietary polyunsaturated fatty acids (PUFAs) such as linoleic acid (18:2n–6) diminished SCD activity (43).

The consequences of regulating the stearoyl-CoA desaturase by PUFAs and cholesterol may be relevant to lipoprotein metabolism. Liver and adipose cell metabolic homeostasis could be dependent on SCD for several reasons. The majority of de novo synthesis of fatty acids in liver is directed toward triglyceride synthesis and secretion (25). The hepatic packaging and secretion of very low density lipoprotein (VLDL) requires synthesis of apolipoprotein B-100 (apoB-100) as well as sufficient amounts of oleic acid which would either come from the diet or from synthesis by SCD (25). Oleic acid is the preferred substrate for acyl-CoA cholesterol acyltransferase, the enzyme responsible for esterification of cholesterol. This esterification prevents the toxic accumulation of free cholesterol in liver and increases the availability of esterified cholesterol for export in the form of VLDL. Repression of SCD activity by PUFAs could lower the amount of cholesterol transported from the liver to the peripheral tissues by limiting the availability of oleic acid or fatty acids in general. Indeed PUFAs mainly of the n–3 series have been shown to exhibit beneficial effects by decreasing plasma lipids and lipoproteins (23). On the other hand, induction of SCD activity by saturated fatty acids and cholesterol would increase plasma lipids and lipoproteins thus leading to cardiovascular disease.

SCD activity has also been shown to be elevated in the adipose tissue of various animal models of obesity (26) and a positive correlation between SCD activity in skeletal muscle and the percentage of body weight has been recently reported in human subjects (27). Mice that lack the obese gene (ob) are characterized by a 5-fold higher deposition of body fat than their lean counterparts. The only consistent change in the fatty acid composition, however, is an increase in palmitoleic acid (16:1n–7) as a result of increased SCD activity (28). In type II diabetes, SCD levels are increased, presumably in response to increased levels of plasma insulin (28).

The regulation of the SCD activity may also affect cancer growth. There is accumulating evidence supporting the hypothesis that a change in C18 fatty acid saturation is important in cancer promotion (29, 30). The higher level of oleic acid in malignant cells accounts for increased membrane fluidity. In general, increased membrane fluidity leads to increased cell metabolism and also higher division rates, features characteristic of cancer cells (29). Stearic acid delays the appearance of spontaneous mammary tumors in mice and growth of carcinogen-induced mammary tumors in rats (30). Stearic acid also inhibits mammary carcinogenesis in vivo (31). On the other hand, increased oleic acid has been reported to be associated with colorectal and mammary tumors (29) and increases malignant cell growth in vitro (31). Thus, one possible mechanism of the anti-cancer activity of certain classes of fatty acids such as sterculic acid and thia fatty acids is to alter the ratio of saturated to monounsaturated fatty acids by reducing SCD activity (32, 33).

Essential fatty acids (EFAs) are required for normal function of mammalian tissues and the lack of EFAs in the diet causes a human and animal disease known as essential fatty acid deficiency (34). In skin it is manifested as a severe scaly lesion accompanied by chronic epidermal hyperproliferation, abnormal differentiation, and abnormal cutaneous barrier function (34). These symptoms are accompanied by the relative abundance of the monounsaturated fatty acids, mainly oleate and palmitoleate, suggesting an increase in SCD activity (34). The severity of the symptoms is lessened upon treatment with linoleic acid, which reduces the levels of C18:1 and C16:1 presumably by reducing SCD activity. Other studies using the human skin model have shown that all-*trans* retinoic acid stimulates the growth of EFA-supplemented keratinocytes while increasing the levels of C16:1 in cells (35), suggesting a role of all-*trans* retinoic acid in the regulation of the SCD in skin diseases.

Regulation of oleic acid synthesis by SCD in the peripheral nervous system has been linked to myelin synthesis (36). During mouse postnatal development, the total SCD activity measured in sciatic nerve homogenates is high and the proportion of oleate rises in sciatic nerve during the first 3 weeks of development. However, this rise in SCD activity and oleate levels does not occur in the demyelinating mutant mouse trembler (36). Oleate is the major fatty acid in the peripheral nervous system (PNS) myelin, comprising between 35 and 45% of the fatty acids of sciatic nerves. Repression of expression of SCD in the PNS could therefore lead to demyelination, a serious neurological disorder.

Immune tissues are susceptible to changes in proliferation and differentiation in response to PUFA. Previous studies have shown stearic acid to be a potent inhibitor of T-lymphocyte proliferation and function (44). The susceptibility of T-lymphocytes to stearate is due to their unique deficiency in SCD activity (44). By contrast, stearate levels do not affect B-lymphocytes because they express an SCD isoform that converts stearate to oleate. Such a fundamental difference between B- and T-lymphocytes indicates a cell type-specific expression with regard to SCD. PUFAs have been shown to repress the expression of the desaturase activity in B-lymphocytes reducing the levels of C18:1 (44). The repression of SCD and subsequent decrease in oleate composition of the lymphocyte membrane could affect B-lymphocyte function.

Thus there is substantial evidence linking SCD activity to a wide range of disorders including obesity, diabetes, cardiovascular disease, skin disease, neurological disorders, and cancer. However, causal relationships between SCD activity and these various disease states remain unclear. Many mechanisms may exist for regulating the activity of SCD by fatty acids and cholesterol in different disease states. Some evidence has been provided that some PUFAs such as sterculic acid and thia fatty acids directly inhibit the SCD activity (32, 33). However, studies of liver, lymphocyte, brain, and adipocytes indicate that the effects

of PUFAs and cholesterol on SCD activity are at the level of gene expression (6, 9, 10, 18, 36, 42, 44–47, 54).

DIETARY n–6 AND n–3 POLYUNSATURATED FATTY ACIDS REPRESS SCD GENE EXPRESSION

In 1978, Jeffcoat and James (43) reported a 60% decrease in hepatic SCD enzyme activity of rats fed a diet containing 60% linoleic acid. This change in enzyme activity we now know is due to altered gene expression (46, 48–54). Two mouse SCD genes (SCD1 and SCD2) are well characterized and are being studied in response to dietary and hormonal changes. At the moment it is not clear why two SCD isoforms exist in the mouse and rat. Under normal dietary conditions the hepatic SCD1 mRNA levels are low. However, when the mice are fed a fat-free high-carbohydrate diet, the SCD1 mRNA is induced about 50-fold (16, 48, 54). This induction is caused by either insulin or carbohydrate (47). The SCD2 gene is not expressed under either dietary condition. The induction of the SCD1 mRNA by a fat-free diet suggested that a fat component present in the chow diet represses the expression of the SCD1 gene in liver. The fat component that represses hepatic SCD1 gene expression has been established to be PUFAs of the n–3 and n–6 series. Thus, when the fat-free diet is supplemented with various triglycerides containing linoleic (18:2n–6), arachidonic (20:4n–6), and linolenic (18:3n–3) acids, SCD1 mRNA expression is repressed whereas triglycerides containing saturated (i.e., C18:0 and C16:0) and monounsaturated fatty acids (C16:1 and C18:1) have very little effect (16). Similar results have been obtained with primary hepatocytes (46). The degree of desaturation was related to repression as 20:4n–6 was more potent than 18:2n–6. In general, as shown in several other studies (55), long chain PUFAs [e.g., 18:2n–6, 18:3n–3, 18:3n–6, 20:4n–6, 20:5n–3, 22:6n–3] repress the expression of the genes that encode lipogenic enzymes including SCD1. The basic requirement for a dietary fatty acid to inhibit expression of lipogenic genes had been proposed to be that it should contain 18 carbons and possess at least 2 conjugated double bonds in the 9 and 12 positions (56). However, recently we have found that the *trans*10, *cis*12 isomer of conjugated linoleic acid also represses SCD mRNA expression in liver (53) but this fatty acid does not contain a double bond at position 9. It contains double bonds at positions 6 and 8 from the ω-carbon. The only double bond position that conjugated linoleic acid and other polyunsaturated fatty acids share is at position 6. The inhibitory effects that conjugated linoleic acid and other polyunsaturated fatty acids have on the expression of the SCD1 mRNA levels may be related to the position and orientation of just one of the double bonds present in all these fatty acids. This information could become important in determining the structure of the polyunsaturated fatty acids that inhibit gene expression.

In adipose tissue of lean and obese Zucker rats, Jones et al. (26) observed a 75% decrease in SCD1 mRNA when both groups were fed a diet high in polyunsaturated fatty acids relative to a control diet. Interestingly, the SCD1 mRNA content was much higher in obese rats compared to normal rats both with and without PUFA supplementation (26). Similar results have been obtained with tissue culture systems as well (26, 52). In the 3T3-L1 adipocyte cell line, arachidonic acid decreased SCD1 mRNA expression in a dose-dependent manner (80% maximum repression), as did linoleic, linolenic, and eicosapentaenoic EPA acids (52). Inhibiting eicosanoid synthesis did not prevent the PUFA suppression of SCD1 gene expression in adipocytes, suggesting that the oxidative metabolism of arachidonic acid to eicosanoids is not involved in the arachidonic acid-mediated decrease of SCD1 mRNA expression in 3T3-L1 adipocytes (52). Furthermore, as indicated above, the desaturase gene expression is repressed by a range of PUFAs, some of which do not give rise to eicosonoids, suggesting that repression of SCD mRNA expression is PUFA-specific.

The regulation of SCD gene expression by PUFAs has also been observed in other tissue types such as brain and the immune tissues. In the brain of neonatal mice, SCD2 mRNA expression is increased in response to feeding diets rich in 18:2n–6 (17). It was suggested that the induction of SCD2 serves to provide oleic acid for the synthesis of myelin (17). In contrast, SCD2 expression in the adult animal is constitutive and not influenced by dietary PUFAs (10, 17). In various lymphoid cells SCD2 mRNA expression is inhibited by 20:4n–6 (18, 44, 45). Stearic acid (18:0) is a potent inhibitor of T-lymphocyte proliferation and function because, in contrast to B-lymphocytes, T-lymphocytes do not express the SCD2 gene (18). However, immature thymocytes and some T-lymphocyte-derived cell lines do express SCD2 mRNA consistent with a developmental down-regulation of the SCD gene within the thymus. SCD1 gene expression, on the other hand, is not detected in any of the lymphoid cells. Such a fundamental difference between B- and T-lymphocytes indicates a cell-type specific gene expression with regard to SCD gene expression. As in adipocytes, the inhibitory effect of 20:4n–6 on SCD2 mRNA expression in B-lymphocytes was independent of arachidonic acid metabolism by either the lipooxygenase or cyclooxygenase pathway. By using protein synthesis inhibitors it was shown that the arachidonic acid-mediated effects on SCD2 mRNA expression were independent of new protein synthesis (45).

The list of tissues in which PUFAs regulate the expression of the SCD genes continues to grow indicating that PUFA action on the SCD genes is much more widespread than originally thought. The differential effects of PUFAs on gene expression may explain the numerous beneficial as well as detrimental effects attributed to various dietary fats in different tissues.

MECHANISMS OF POLYUNSATURATED FATTY ACID CONTROL OF SCD GENE EXPRESSION

A. Transcriptional control

The molecular mechanisms by which PUFAs regulate SCD gene expression in different tissues are still poorly

understood, although progress has been made in the last few years. PUFA-mediated suppression of SCD1 expression in liver and primary hepatocytes and of SCD2 expression in lymphocytes was shown to be largely due to a decrease in their rates of gene transcription (16, 45, 46, 48–51, 54, 57). Therefore, many recent studies have been predicated on the hypothesis that a *cis*-acting PUFA responsive element (PUFA-RE) exists in the promoters of SCD genes to which a transcription factor binds, thus blocking transcription. Using deletion analysis, we localized the SCD1 and SCD2 PUFA-REs to a 60 bp region in each of their promoters (57). This is the only region of high sequence homology within the promoters of the two SCD genes as depicted in Fig. 2. The PUFA-RE of the rat S14 and pyruvate kinase genes have also been mapped (55, 56, 58, 59) and were found to share homologous sequences. However, these PUFA-REs are not homologous to those of SCD1 or SCD2.

PUFAs are known to activate nuclear transcription factors termed peroxisome proliferator-activated receptors (PPARs) and have, therefore, been hypothesized to be the endogenous activators of this receptor (56). Both peroxisome proliferators and PUFA (56, 60) repress lipogenic genes, such as S14 and fatty acid synthase. However, the PUFA suppression of these genes does not require peroxisome proliferator activator receptors (61, 62). Peroxisome proliferators and polyunsaturated fatty acids in adipocytes (63, 64) also repress the GLUT4 gene expression. It was speculated that the PUFA-RE in the GLUT4 gene is related to the peroxisome proliferator responsive element (PPRE). We hypothesized that PUFA-activated PPAR, along with its heterodimeric partner retinoic acid X receptor (RXR), might repress SCD1 gene transcription. Unexpectedly, our studies (49) on the effects of peroxisome proliferators on SCD1 gene expression showed that PUFA and peroxisome proliferators had opposing effects on the SCD1 mRNA levels in mouse liver. Unlike the S14 and fatty acid synthase genes, peroxisome proliferators induced the expression of the SCD gene. In addition, transient transfection experiments localized the SCD1 PPRE to an area of the SCD1 promoter that is distinct from the PUFA-RE (49). This indicates that different mechanisms account for the transcriptional regulation of the SCD1 gene by peroxisome proliferators and PUFA and suggests the existence of a putative PUFA binding protein (54).

The search for a PUFA-specific transcription factor for SCD gene repression has now become a major focus in our laboratory. We demonstrated the binding of nuclear proteins to the PUFA-RE of the SCD1 and SCD2 genes by DNA mobility shift analysis (57). Two of the proteins that bind to specific regions within the PUFA-RE of the SCD genes have been identified by Tabor and coworkers (65) and they are the sterol regulatory element binding proteins (SREBPs) and the CCAAT-binding factor/nuclear factor Y or NF-Y. While the expression vectors containing the cDNAs corresponding to SREBP-1 and SREBP-2 activate the expression of the SCD genes both in vitro and in vivo (65, 66–69), the SREBPs do not by themselves seem to directly mediate PUFA repression of the SCD genes (see below). Our current model indicates that PUFAs bind to a putative PUFA-binding protein (PUFA-BP) and repression of transcription occurs upon the binding of the PUFA-BP to the PUFA-RE of the SCD genes (**Fig. 3**). Crosstalk between the putative PUFA binding protein and the SREBP or NFY-1 is also suggested.

B. Post-transcriptional control

Studies on the SCD genes in mature adipocytes and in yeast have shown that the effect of PUFA on these genes could be at the level of mRNA stability (52, 70). When added to cultures of fully differentiated 3T3-L1 adipocytes, arachidonic acid decreased the SCD1 mRNA half-life from 8 h to 4 h (52). By contrast, oleic acid and stearic acid did not affect SCD1 mRNA stability. Therefore, this response is unique to PUFAs. Arachidonic acid also decreased the stability of the SCD2 transcript (A. Sessler and J. M. Ntambi, unpublished results). Although transcriptional regulation could not be completely ruled out, changes in transcriptional rates were not detected, suggesting that transcriptional regulation does not play a significant role in PUFA suppression of adipocyte SCD gene expression in mature adipocytes (52). The observed reduction in enzyme activity (60%) could be completely accounted for by decreases in SCD1 mRNA levels (80%). Thus, there appeared to be no additional down-regulation occurring post-translationally. Therefore, in contrast to what occurs in hepatocytes, changes in mRNA stability are the major determinant of SCD1 mRNA abundance in adipocytes in response to PUFA.

Destabilization of SCD mRNA in adipocytes may be regulated through mRNA sequences in the 3′-untranslated region (UTR). The mouse, rat, and human SCD cDNAs contain an unusually long 3′-UTR (9–12, 71). The role of such a long 3′-noncoding stretch is currently unknown, though it contains several structural motifs (e.g., AUUUA) characteristic of mRNA destabilization sequences (52). Four of these sequences are clustered close to the 3′-end of the coding region. Because these AU-rich elements (ARE) play active roles in the selective degradation of several mRNAs in response to various factors, these sequences could be possible targets of PUFA effects on SCD1 and SCD2 mRNA in adipocytes. In yeast, PUFAs act through sequences in the 5′-UTRs to decrease the OLE1 mRNA stability (70). Northern blot analysis shows that the human SCD gene gives rise to two mRNA transcripts of 3.9 and 5.2 kb which arise as a consequence of the two polyadenylation signals, indicating that the two differently expressed transcripts encode the same SCD polypeptide (12). The function of the polyadenylation is not known but could be in addition to the transcriptional control, a means by which the two transcripts differ in stability or translatability thus allowing for rapid and efficient changes in cellular environment (12).

The work on SCD gene regulation by PUFA in adipocytes suggests that PUFA regulate SCD gene expression through different mechanisms in different tissue types, the reasons for which are not yet understood. However, SCD gene provides a good model to study the effects of

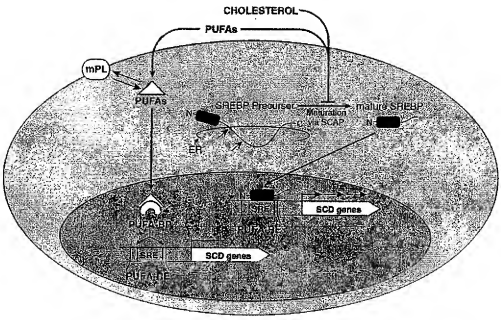

Fig. 3. Pathways of SCD regulation by PUFA and cholesterol. PUFA and cholesterol repress the maturation of the sterol regulatory element binding protein (SREBP) via the SREBP cleavage-activating protein (SCAP) that would otherwise translocate from the endoplasmic reticulum (ER) into the nucleus to activate the transcription of the SCD genes by binding to the sterol regulatory element (SRE). PUFAs either from the diet or membrane phospholipid (mPL) independently bind the putative binding protein (PUFA-BP) and the PUFA and PUFA-BP complex represses transcription of the SCD genes by binding to the polyunsaturated fatty acid response element (PUFA-RE). Crosstalk between the two pathways is designated by the dashed arrow.

PUFA on mRNA stability. The ongoing search in our lab and others for possible protein mediators that destabilize SCD1 mRNA should provide further definition to the molecular basis of PUFA regulation of lipogenic gene expression.

CHOLESTEROL AS A REGULATOR OF SCD GENE EXPRESSION

Nearly two decades ago, Chin and Chang (72) demonstrated that there was an increase in the SCD enzyme activity when Chinese hamster ovary cells were incubated with lipid-depleted media. They noted that the desaturase activity was reduced when the medium was supplemented with cholesterol. Subsequently, mutants were isolated that failed to induce enzymes involved in both cholesterol and monounsaturated fatty acid biosynthesis. Revertants were able to synthesize both cholesterol and monounsaturated fatty acids concomitantly. Using differential display techniques to identify genes that are transcriptionally activated by sterol regulatory element binding proteins (SREBPs), Tabor et al. (65) recloned the SCD2 cDNA from Chinese hamster ovary cells and demonstrated that the SCD2 mRNA expression is repressed in these cells when cultured in the presence of cholesterol. These in vitro studies using the Chinese hamster ovary cells showed that, like PUFAs, cholesterol represses the expression of the SCD genes and enzyme activities.

There are in vivo rat studies, however, that show that contrary to results in vitro, high levels of cholesterol can induce SCD gene expression. In one study, feeding rats with various diets supplemented with cholesterol increased SCD enzyme activity and SCD mRNA levels in liver (40–42). The increase in SCD enzyme activity correlated with increased synthesis of oleic acid and its enhanced incorporation into cholesteryl esters. Another study considered the expression of the SCD mRNA in LXRα receptor knockout mice (73). LXRα is the oxysterol receptor that mediates regulation of gene expression by cholesterol or its metabolites (73, 74). Mice lacking LXRα receptor expressed low levels of SCD mRNA in the presence or absence of cholesterol feeding while the wild-type animals fed 2% cholesterol had their SCD mRNA levels up-regulated 4-fold (72). As expected, the mRNA levels corresponding to several enzymes involved in cholesterol biosynthesis were up-regulated in the LXRα knockout animals and down-regulated in the wild-type animals upon cholesterol feeding. These in vivo studies would be consistent with the possibility that the liver, when challenged with excess cholesterol, increases SCD activity in order to provide oleoyl-CoA as a substrate for cholesterol esterification and storage to package into VLDL for secretion and transport to other tissues. As esterified cholesterol does not down-regulate the LDL receptor, the liver can continue cholesterol uptake and prevent hypercholesterolemia. It is not clear from the in vivo studies whether cholesterol or its metabolites induce the transcription of the SCD1 through the LXRα or indirectly

through the regulation of the SREBPs. It is not known whether the induction of SCD gene expression by cholesterol is liver-specific or occurs in other tissues as well.

There are other studies that support cholesterol's regulation of SCD expression in vivo. Female mice express higher levels of SCD mRNA than male mice (75). The underlying mechanistic basis for the higher levels of expression of SCD1 gene in female mice is unknown but it could be due to differences in levels of hormones such as estrogen and testosterone which are synthesized from cholesterol. Estrogen administration causes a remarkable increase of plasma lipids in mice and of VLDL in avian species (76). Androgens stimulate the expression of the stearoyl-CoA desaturase in the sebaceous glands of hamsters (77). Estrogen has also been shown to induce SCD activity in rooster liver (78) but whether this occurs at the level of gene expression has not been established. Several peroxisome proliferators induce SCD activity differently in males than in females and this difference has been shown to be due to higher levels of testosterone in males (79). Thus, the differences in SCD1 gene expression may aid our understanding of how certain gender-related diseases such as cancer, diabetes, obesity, and heart disease are influenced by dietary fat lipid saturation and steroid hormones.

TRANSCRIPTIONAL REGULATION OF SCD GENES BY STEROL REGULATORY ELEMENT BINDING PROTEINS (SREBPs)

Transcriptional activation of genes containing sterol responsive element (SRE) is known to be under the regulation of sterols through modulation of the proteolytic maturation of the sterol responsive element binding proteins (SREBP-1 and SREBP-2) (80, 81). The SREBPs are inserted into the membrane of the endoplasmic reticulum envelope in a wide variety of tissues. In sterol-deficient cells, proteolytic cleavage of SREBPs releases their N-terminal mature forms from the membrane, enabling them to enter the nucleus, where they bind to the SREs and activate genes involved in cholesterol, triglyceride, and fatty acid biosynthesis (66–69). In the presence of sterols, the proteolytic process is inhibited and the transcription of the genes is reduced because little SREBP is available to activate their transcription.

Tabor and coworkers (65) showed that the transcription of the mouse SCD2 is regulated in response to alterations in either levels of sterols or the levels of nuclear SREBP/ADD1. Increased expression/nuclear localization of SREBP-1 and SREBP-2 after incubation of cells in sterol-depleted medium was sufficient to enhance transcription of the SCD2 gene and resulted in increased SCD2 mRNA levels. The observation that hepatic SCD1 and SCD2 mRNA levels were induced in transgenic mice that overexpress SREBP-1 and SREBP-2 (67–70), leading to increased synthesis of monounsaturated fatty acids, is consistent with the direct transcriptional activation of the SCD1 promoter by SREBPs. The in vivo experiments with LXRα knockout mice alluded to above suggest that LXRα is involved in the activation of SCD gene transcription by cholesterol or its metabolites. This is similar to the activation of the gene encoding cholesterol 7α-hydroxylase, a rate-limiting enzyme in bile acid biosynthesis (74). In the LXRα knockout mouse it was also shown that SREBP-1 mRNA and protein levels are reduced and that SREBP-1 mRNA was induced only in wild-type animals fed 2% cholesterol in their diets, suggesting that cholesterol regulates the expression of SREBP through LXRα. If this is the case, then the in vivo experiments would imply that LXRα indirectly regulates the SCD expression by controlling SREBP-1 expression. This phenomenon would be consistent with studies of the transgenic mice expressing a constitutively active form of SREBP-1 in which fatty acid-synthesizing enzymes are dramatically elevated resulting in a fatty liver phenotype (67). These studies would also be consistent with the in vitro results that show activation of the SCD gene expression by SREBPs.

A novel sequence in the PUFA-RE of the SCD2 gene that functions as an SRE has been identified (65). This sequence (5′AGCAGATTGTG3′), shown to bind purified SREBP, is distinct from previously described SREs. It does not contain the direct repeat nor does it contain a functional E-box (65), but within its vicinity are two CCAAT boxes for the binding of NF-Y (Fig. 2). SREBP and NF-Y have been shown to dimerize in the activation of the sterol-dependent transcription of several sterol-regulated genes (81–84) and it is more than likely that both transcription factors are required in cholesterol regulation of the SCD genes.

PUFAs and oleic acid have been shown to reduce the expression of promoters with sterol regulatory elements by inhibiting the proteolytic maturation of SREBP (85, 86). These studies suggest that the SREBP participates in the repression of the SCD gene transcription by PUFA. In addition, oleic acid has been shown to potentiate the maturation of SREBP by sterols in CHO-K1 cells (86). However, oleic acid could not reduce the transcription of the SCD genes both in vivo and in vitro (16, 45, 57). Thus, SREBP maturation does not seem to exhibit the selectivity required to explain PUFA control of SCD gene transcription. Transfection of the SCD2 promoter constructs containing the PUFA-RE linked to a reporter gene in HepG2 cells results in repression of reporter gene expression in the presence of cholesterol and PUFAs (65). However, the SREBP or the NF-Y elements on their own do not mediate PUFA repression in a heterologous context (57) but PUFA repression is observed only when the entire 60 bp PUFA-RE is used in a heterologous context (57). Therefore, although there are indications that SREBP maturation as well as binding of the SREBP and NF-Y to the PUFA-RE are involved in PUFA repression, there is strong evidence for the existence of an SREBP-independent mechanism involving a putative PUFA-binding protein (Fig. 3) through which PUFAs repress SCD gene expression. Crosstalk could exist between the SREBP, NF-Y, and the putative PUFA binding protein. More research is required in this area to resolve this issue.

CONCLUSION AND FUTURE DIRECTION

Research in the past few years has confirmed the importance of PUFAs and cholesterol as universal cellular regulators. The discovery that lipids can affect gene transcription, and thus modulate the cell's metabolic state, is essential to our understanding of responses to dietary changes. Both PUFAs and cholesterol can control the synthesis of monounsaturated fatty acids in liver by regulating the expression of the SCD genes. PUFAs regulate expression of SCD in other tissues as well. Whether cholesterol regulates SCD gene expression in tissues other than the liver needs further investigation. The role of SREBP in mediating the action of cholesterol is well established through several elegant studies but its role in the regulation of gene expression by PUFA needs more study. It is very interesting that the SCD PUFA-RE and SRE overlap, but our current understanding of SCD gene expression suggests an SREBP-independent pathway of PUFA repression of gene expression. Several lipogenic genes such as FAS and S14 are also regulated by PUFA and cholesterol but the regulation of the SCD gene assumes greater importance when we consider the multitude of human diseases in which abnormal synthesis of monounsaturated fatty acids is a factor. Further elucidation of the mechanisms by which PUFA and cholesterol alter cellular monounsaturated fatty acids is therefore needed to understand the impact of SCD gene regulation in various human disease states. ■

I thank Victor Munsen, Dr. Dale Hereld, and Dr. Peter Edwards for useful discussions. Research reported in this work was supported by USDA Hatch grant # 3784 and support from the Graduate School of the College of Agriculture and Life Sciences of the University of Wisconsin-Madison and department of Biochemistry of the University of Wisconsin-Madison.

Manuscript received 31 March 1999 and in revised form 27 May 1999.

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Targeted Disruption of Stearoyl-CoA Desaturase1 Gene in Mice Causes Atrophy of Sebaceous and Meibomian Glands and Depletion of Wax Esters in the Eyelid[1]

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ABSTRACT Stearoyl-CoA desaturase (SCD) is a microsomal rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids (MUFA), mainly oleate (18:1) and palmitoleate (16:1), which are the major MUFA of membrane phospholipids, cholesterol esters and triglycerides. Three well-characterized isoforms of SCD, SCD1, SCD2 and SCD3, exist in mice. To investigate the physiologic functions of SCD1, we generated SCD1 null (SCD1−/−) mice. The skin and eyelid of SCD1−/− mice are deficient in triglycerides and cholesterol esters, and the eyelid also is deficient in wax esters. Furthermore, the eyelid and skin of SCD1−/− mice have higher levels of free cholesterol. SCD1−/− mice develop cutaneous abnormalities and narrow eye fissure with atrophic sebaceous and meibomian glands. Consumption of diets containing high levels of oleate, failed to restore the levels of triglycerides, cholesterol esters and wax esters in SCD1−/− mice to the levels found in the eyelid of wild-type mice. These results reveal a physiologic role of SCD in cholesterol homeostasis as well as in the de novo biosynthesis of cholesterol esters, triglycerides and wax esters required for normal skin and eyelid function. J. Nutr. 131: 2260–2268, 2001.

KEY WORDS: • *stearoyl-CoA desaturase* • *meibomian gland* • *sebaceous gland* • *wax ester* • *eyelid* • *mice*

Stearoyl-CoA desaturase (SCD)[3] is a rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids (MUFA). It catalyzes the Δ9-*cis* desaturation of acyl-CoA substrates; the preferred substrates are palmitoyl-CoA and stearoyl-CoA, which are converted to palmitoleyl-CoA and oleoyl-CoA, respectively (1). The resulting MUFA are substrates for incorporation into membrane phospholipids, triglycerides and cholesterol esters. Studies in mice and rats have shown that SCD expression is highly regulated by diet, hormonal factors, developmental processes, temperature, metals, alcohol, peroxisomal proliferators and phenolic compounds, resulting in the alteration of the fatty acid composition of membrane phospholipids, triglycerides and cholesterol esters (2). Effects on the composition of membrane phospholipids ultimately determine membrane fluidity, whereas the effects on the composition of cholesterol esters and triglycerides can affect lipoprotein metabolism and adiposity. Thus, the regulation of SCD is of considerable physiologic importance, and alteration in SCD activity has been implicated in a wide range of disorders including diabetes, atherosclerosis, cancer and obesity (2–4).

A number of mammalian SCD genes have been cloned. Two SCD genes have been cloned in rats and four have been cloned from mice, three of which (SCD1, SCD2 and SCD3) are well characterized (3,5–8). A single human SCD gene that is highly homologous to the rat and mouse SCD genes was cloned and characterized (9). Despite the fact that the mouse, rat and human SCD genes are structurally similar, sharing ~87% nucleotide sequence identity in the coding regions, some portions of their 5′ flanking regions differ, resulting in divergent tissue-specific gene expression. In some mouse tissues such as the skin, the three SCD gene isoforms are expressed (8). The physiologic importance of having two or more SCD isoforms expressed in the same tissue is not currently known but could be related to the substrate specificity of each SCD isoform or the means by which cells compartmentalize lipid biosynthesis for specific functions (10).

The existence of multiple SCD genes in mouse and rat tissues makes it difficult to determine the role of each gene in lipid metabolism. Most previous studies have assessed SCD gene function by measuring mRNA expression but have not differentiated which SCD isoform is responsible for the altered total SCD activity. A clue to the physiologic role of the SCD1 gene and its endogenous products (MUFA) has come from recent studies of the asebia (ab^1) mutant mouse strain (11), which has an extensive natural deletion in the SCD1 gene. Another mutant strain (ab^{2J}), which shows a splice junction deletion of exon 2 in the SCD1 gene and is allelic with the ab^1,

[1] Supported by American Heart Association and by NIEHS-National Institutes of Health training grant ES09090-04 to the University of Wisconsin-Madison EHS Center for Developmental & Molecular Toxicology.

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[3] Abbreviations used: ACAT, acyl-CoA:cholesterol acyl transferase enzyme; apo, apolipoprotein; ECL, enhanced chemiluminescence; GLC, gas-liquid chromatography; HPTLC, high performance TLC; MUFA, monounsaturated fatty acids; PCR, polymerase chain reaction; SCD, stearoyl-CoA desaturase; TBS, Tris buffered saline; WAT, white adipose tissue; +/+, wild-type; +/−, heterozygous; −/− homozygous.

0022-3166/01 $3.00
Manuscript received 5 April 2001. Initial review completed 15 May 2001. Revision accepted 23 June 2001.

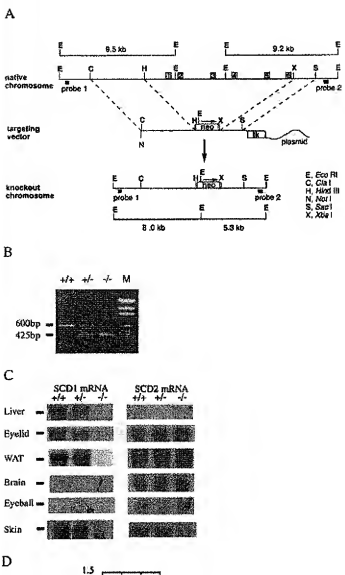

FIGURE 1 Generation of the SCD1 null mice. (*A*) Targeting strategy for disruption of the SCD1 gene. A neomycin-resistant cassette replaced the 6 exons of the gene by homologous recombination, resulting in the replacement of the complete coding region of the SCD1 gene. Gene-targeting events were verified by Southern blot analysis using EcoRI and probe 1 or 2 or by polymerase chain reaction (PCR) analysis. (*B*) PCR analysis of DNA isolated from different genotypes of F1 offspring using appropriate primers. The 425-bp fragment detected represents the mutated allele. In breeding heterozygotes, wild type, heterozygotes and homozygotes were born in Mendelian fashion (+/+:+/-:-/- = 21:43:20). (*C*) Northern blot analysis for the expression of SCD1 and 2 mRNA. Total RNA (20 μg) isolated from the liver, eyelid, white adipose tissue (WAT), brain, eyeball and skin of wild-type, SCD1+/– and SCD1–/– mice pooled from 5 mice of each group was subjected to Northern blot analysis followed by hybridization with labeled probes specific for SCD1 and SCD2 cDNA. A cDNA probe for pAL15 (16) was used to confirm equal loading. (*D*) SCD enzyme activity has recently been described (12). The homozygous ab[3J] SCD1 mutant mouse is similar to the ab[J] mouse except for the extent of epidermal thickness, scaling and epidermal permeability barrier as measured by transepidermal water loss methods (12). The basis for the difference in phenotype between these two mutant mouse strains is not presently understood but has been attributed to modification of gene effects by strain background (12). To further explore the physiologic roles of the SCD gene isoforms, we generated mice with a targeted disruption of the SCD1 gene. Our results indicate that SCD1 plays a major role in the de novo synthesis of triglycerides, cholesterol esters and wax esters required for normal skin and eyelid function.

MATERIALS AND METHODS

Generation of the SCD1 knockout mice. Mouse genomic DNA for the targeting vector was cloned from the 129/SV genomic library. The targeting vector construct was generated by insertion of a 1.8-kb Xba I/Sac I fragment with 3′ homology as a short arm and 4.4-kb Cla I/Hind III fragment with 5′ homology cloned adjacent to the neo expression cassette. The construct also contains a herpes simplex virus thymidine kinase cassette 3′ to the 1.8-kb homology arm allowing positive/negative selection. The targeting vector was linearized by Not I and electroporated into embryonic stem cells. Selection with geneticin and gancyclovior was performed. The clones resistant to both geneticin and gancyclovior were analyzed by Southern blot after EcoR1 restriction enzyme digestion and hybridized with a 0.4-kb probe located downstream of the vector sequences. For polymerase chain reaction (PCR) genotyping, genomic DNA was amplified with primer A (5′-GGGTGAGCATGGTGCTCAGTCCCT-3′), which is located in exon 6, primer B (5′-ATAGCAGGCATGCTGGGAGAT-3′), which is located in the neo gene (425-bp product, targeted allele) and primer C (5′-CACACCATATCTGTCCCCGACAAATGTC-3′), which is located downstream of the targeting gene (600-bp product, wild-type allele). PCR conditions were 35 cycles each of 45 s at 94°C, 30 s at 62°C and 1 min at 72°C. The targeted cells were microinjected into C57Bl/6 blastocysts to generate the chimeric mice. The chimeric mice were then crossed with 129/SvEv-Taconic females to generate SCD1–/– mice having a genetic background of SV129. Mice were maintained on a 12-h dark/light cycle and were fed a nonpurified diet (5008 test diet; PMI Nutrition International, Richmond, IN; hyyp://www.labdiet.com/5008.htm) or semipurified diets containing 5 g/100 g soybean oil (control diet) or a high oleate oil (high 18:1 diet) (4). The semipurified diet was purchased from Harlan Teklad (Madison, WI) and contained (per 100 g diet): 20 g vitamin-free casein, 5.0 g soybean oil or high oleate oil, 0.35 g L-cystine, 13.2 g Maltodextrin, 51.7 g sucrose, 5.0 g cellulose, 3.5g mineral mix (AIN-93) (13), 1.0 g vitamin mix (AIN-93) (13) and 0.3 g choline bitartrate. The fatty acid composition of the experimental oils was determined by gas-liquid chromatography (GLC). The soybean oil contained (per 100 g oil): 11 g palmitic acid (16:0), 23 g oleic acid [18:1(n-9)], 53 g linoleic acid [18:2(n-6)] and 8 g linolenic acid [18:3(n-3)]. The high oleate oil contained (per 100 g oil): 7 g 16:0, 50 g 18:1(n-9), 35 g 18:2(n-6) and 5 g 18:3(n-3). Homozygous (SCD1–/–), heterozygous (SCD1+/–) and wild-type (SCD1+/+) mice were housed and bred in a pathogen-free barrier facility of the department of Biochemistry operating on a 12-h light:

and immunoblot analysis of SCD from livers of wild-type, SCD1+/– and SCD1–/– mice. For enzyme activity, aliquots of microsome fraction (100 μg) from livers of each group were incubated with a reaction mixture containing [1-^{14}C]stearoyl-CoA for 5 min. The products were saponified and acidified and the fatty acids were extracted and separated by TLC. Enzyme activity is represented as nanomoles of substrate desaturated per milligram of protein per minute. Data are denoted as the mean ± so (n = 3). For immunoblot analysis, aliquots of membrane fraction (80 μg) from pooled livers of each group were subjected to 10% SDS polyacrylamide gel electrophoresis followed by detection with SCD antibody.

TABLE

Fatty acid composition of several tissues from SCD+/+,

Fatty acids	14:0	16:0	16:1(n-7)	18:0	18:1(n-9)	18:1(n-7)
	g/100 g fatty acids					
Liver						
+/+	0.6 ± 0.1	25.9 ± 1.1	1.1 ± 0.2[a]	16.1 ± 1.6[b]	16.2 ± 0.5[a]	1.7 ± 0.2[a]
+/−	1.0 ± 0.1	26.6 ± 0.2	0.8 ± 0.0[a]	17.8 ± 2.2[ab]	13.8 ± 0.3[b]	1.4 ± 0.2[a]
−/−	1.0 ± 0.4	27.2 ± 0.3	0.5 ± 0.1[b]	22.8 ± 0.3[a]	10.6 ± 1.3[c]	1.0 ± 0.1[b]
Eyelid						
+/+	3.2 ± 0.3	32.0 ± 0.3[b]	3.3 ± 0.5[a]	25.4 ± 0.8[b]	20.7 ± 1.1[a]	3.3 ± 0.2[a]
+/−	3.3 ± 0.3	33.8 ± 1.6[ab]	2.3 ± 0.1[a]	27.1 ± 2.6[ab]	18.6 ± 1.2[ab]	2.7 ± 0.1[b]
−/−	3.3 ± 0.1	34.2 ± 0.4[a]	1.3 ± 0.2[b]	28.5 ± 0.5[a]	17.6 ± 0.8[b]	1.3 ± 0.4[c]
White adipose tissue						
+/+	3.3 ± 0.8	27.5 ± 0.8	5.2 ± 0.2[a]	5.6 ± 0.1[c]	35.0 ± 0.5[a]	1.9 ± 0.0
+/−	3.1 ± 0.8	29.6 ± 1.8	3.5 ± 0.9[a]	6.8 ± 0.3[b]	31.7 ± 0.9[b]	1.7 ± 0.2
−/−	2.7 ± 1.0	28.9 ± 1.5	1.5 ± 0.4[b]	14.6 ± 3.5[a]	28.8 ± 0.2[c]	1.7 ± 0.1
Skin						
+/+	3.0 ± 0.9	29.8 ± 0.9	3.8 ± 1.6[a]	9.8 ± 1.3[b]	31.8 ± 1.1[a]	2.1 ± 0.1[a]
+/−	2.9 ± 0.2	29.6 ± 0.1	2.2 ± 0.4[b]	13.0 ± 0.1[a]	28.0 ± 2.3[ab]	2.0 ± 0.0[a]
−/−	3.1 ± 0.0	31.3 ± 1.4	1.6 ± 0.3[b]	15.3 ± 2.0[a]	27.6 ± 0.3[b]	1.8 ± 0.0[b]
Brain						
+/+	1.1 ± 0.1	26.6 ± 1.5	0.9 ± 0.2	23.0 ± 2.5	17.2 ± 1.2	3.1 ± 0.1
+/−	1.0 ± 0.3	27.6 ± 0.8	0.8 ± 0.1	23.0 ± 2.8	17.6 ± 1.0	3.4 ± 0.4
−/−	1.1 ± 0.1	26.7 ± 0.9	0.8 ± 0.0	22.7 ± 2.8	17.0 ± 2.1	3.3 ± 0.2
Eyeball						
+/+	2.9 ± 0.2	30.5 ± 0.8	1.5 ± 0.1	27.8 ± 1.6	18.6 ± 1.5	2.6 ± 0.9
+/−	2.9 ± 0.1	31.5 ± 1.0	1.3 ± 0.2	27.8 ± 1.1	18.1 ± 0.4	2.2 ± 0.6
−/−	3.1 ± 0.1	31.5 ± 1.2	1.3 ± 0.2	29.3 ± 2.0	18.3 ± 0.7	2.2 ± 0.7

[1] Values are means ± SD, $n = 3$. Values in column for a tissue with different letters are significantly different ($P < 0.05$, one-way ANOVA).
[2] The mice were 3-mo-old males. Only the major fatty acids were presented.
[3] Abbreviations: +/+, wild-type mice; +/−; SCD1 heterozygous mice; −/−, SCD1 homozygous mice.

dark cycle. The breeding of these mice is in accordance with the protocols approved by the animal care research committee (ACRC) of the University of Wisconsin-Madison.

Materials. Radioactive [α-^{32}P]dCTP (111 TBq/mmol) was obtained from Dupont (Wilmington, DE). TLC plates (TLC Silica Gel G60) were from Merck (Darmstadt, Germany). [1-^{14}C]-stearoyl-CoA was purchased from American Radiolabeled Chemicals (St Louis, MO). Immobilon-P transfer membranes were from Millipore (Danvers, MA). Enhanced chemiluminescence (ECL) Western blot detection kit was from Amersham-Pharmacia (Piscataway, NJ). All other chemicals were purchased from Sigma (St Louis, MO).

Lipid analysis. Total lipids were extracted from tissues according to the method of Bligh and Dyer (14), and phospholipids, wax esters, free cholesterol, triglycerides and cholesterol esters were separated by silica gel high performance TLC (HPTLC). Petroleum hexane/diethyl ether/acetic acid (80:30:1, v/v/v) or benzene/hexane (65:35, v/v) was used as a developing solvent (15). Spots were visualized by 5.0 mmol/L 2′,7′-dichlorofluorescein in 95% ethanol or by 0.63 mol/L cupric sulfate containing 0.89 mol/L phosphoric acid. The wax ester, cholesterol ester and triglyceride spots were scraped, 1 mL of 5% HCl-methanol added and heated at 100°C for 1 h (4). The methyl esters were analyzed by GLC using cholesterol heptadecanoate, triheptadecanoate and heptadecanoic acid as internal standards. Free cholesterol (Free Choleterol C, Wako Chemicals, Japan), total cholesterol (Cholesterol CII, Wako) and triglyceride [Triglycerides (INT) 20, Sigma] contents of eyelid and skin were determined by enzymatic assays.

Isolation and analysis of RNA. Total RNA was isolated from livers using the acid guanidinium-phenol-chloroform extraction method (16). Total RNA (20 μg) was separated by 1.0% agarose/2.2 mol/L formaldehyde gel electrophoresis and transferred onto nylon membrane. The membrane was hybridized with ^{32}P-labeled SCD1 and SCD2 probes. A pAL15 probe was used as control for equal loading (4).

SCD activity assay. Stearoyl-CoA desaturase activity was measured in liver microsomes essentially as described (17). Tissues were homogenized in 10 volumes of 100 mmol/L potassium buffer, pH 7.4. The microsomal membrane fractions (100,000 × g pellet) were isolated by sequential centrifugation. Reactions were performed at 37°C for 5 min with 100 μg of protein homogenate and 60 μmol/L of [1-^{14}C]-stearoyl-CoA (60,000 dpm), 2 mmol/L of NADH and 100 mmol/L of Tris/HCl buffer (pH 7.2). After the reaction, fatty acids were extracted and then methylated with 5% HCl/methanol. Saturated fatty acid and MUFA methyl esters were separated by 100 g/L $AgNO_3$-impregnated TLC using hexane/diethyl ether (9:1) as developing solution. The plates were sprayed with 5.0 mmol/L 2′,7′-dichlorofluorescein in 95% ethanol and the lipids were identified under UV light. The fractions were scraped off the plate, and the radioactivity was measured using a liquid scintillation counter. The enzyme activity was expressed as nmol/(min · mg protein).

Immunoblotting. Pooled liver membranes from three mice of each group were prepared as described (18). The same amount of protein (25 μg) from each fraction was subjected to 100 g/L SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membranes at 4°C. After blocking with 10 g/100 mL nonfat milk in Tris buffered saline buffer (pH 8.0) plus Tween at 4°C overnight, the membrane was washed and incubated with rabbit anti-rat SCD as primary antibody and goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate as the secondary antibody. Visualization of the SCD protein was performed with an ECL Western blot detection kit (Amersham-Pharmacia).

Histology. Tissues were fixed with neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin as described (19).

Statistical analysis. Stastistical analysis of the data was carried out using StaView (Abacus Concepts, Berkeley, CA). Data were analyzed using Student's t test or one-way ANOVA followed by

1

SCD+/− and SCD−/− mice-fed a semipurified diet[1,2,3]

18:2(n-6)	20:4(n-6)	22:6(n-3)	16:1(n-7)/16:0	18:1(n-9)/18:0	18:1(n-7)/18:0
			g/g		
16.3 ± 4.2	9.2 ± 0.8[a]	7.8 ± 0.3	0.042 ± 0.010[a]	1.018 ± 0.151[a]	0.107 ± 0.008[a]
14.8 ± 3.2	9.2 ± 1.0[a]	8.9 ± 1.0	0.031 ± 0.001[b]	0.782 ± 0.121[b]	0.080 ± 0.021[ab]
13.9 ± 2.9	6.8 ± 1.6[b]	8.8 ± 1.0	0.019 ± 0.004[b]	0.466 ± 0.053[b]	0.043 ± 0.003[b]
5.2 ± 0.4[b]	2.8 ± 0.0	4.1 ± 0.4	0.102 ± 0.015[a]	0.814 ± 0.019[a]	0.128 ± 0.003[a]
5.3 ± 0.6[b]	2.7 ± 0.1	4.2 ± 0.3	0.069 ± 0.002[a]	0.690 ± 0.023[b]	0.101 ± 0.008[b]
7.1 ± 0.3[a]	2.8 ± 0.5	3.9 ± 0.3	0.037 ± 0.005[b]	0.619 ± 0.017[c]	0.045 ± 0.013[c]
19.5 ± 0.9	0.3 ± 0.1	0.2 ± 0.1[b]	0.190 ± 0.012[a]	6.211 ± 0.215[a]	0.340 ± 0.005[a]
18.0 ± 2.6	0.4 ± 0.2	0.2 ± 0.0[b]	0.119 ± 0.025[b]	4.653 ± 0.322[b]	0.248 ± 0.036[b]
18.5 ± 1.6	0.4 ± 0.0	0.8 ± 0.0[a]	0.050 ± 0.012[c]	2.050 ± 0.501[c]	0.122 ± 0.039[c]
15.5 ± 0.8[b]	0.9 ± 0.0	0.7 ± 0.1	0.127 ± 0.017[a]	3.237 ± 0.433[a]	0.212 ± 0.036[a]
15.3 ± 0.9[b]	1.1 ± 0.2	1.1 ± 0.3[a]	0.075 ± 0.012[b]	2.154 ± 0.169[b]	0.156 ± 0.001[b]
17.6 ± 1.3[a]	0.9 ± 0.0	0.8 ± 0.0	0.050 ± 0.007[c]	1.814 ± 0.119[b]	0.128 ± 0.015[b]
1.1 ± 0.1	8.4 ± 1.6	11.7 ± 1.8	0.034 ± 0.004	0.748 ± 0.028	0.135 ± 0.017
1.1 ± 0.0	8.1 ± 1.8	11.0 ± 2.2	0.029 ± 0.003	0.769 ± 0.049	0.149 ± 0.034
1.2 ± 0.1	8.5 ± 1.8	11.9 ± 2.1	0.030 ± 0.000	0.751 ± 0.002	0.141 ± 0.023
2.6 ± 0.0	2.8 ± 0.3	7.8 ± 1.3	0.051 ± 0.002	0.672 ± 0.089	0.091 ± 0.028
2.6 ± 0.0	2.5 ± 0.5	6.9 ± 0.1	0.042 ± 0.006	0.652 ± 0.040	0.077 ± 0.020
2.4 ± 0.4	2.3 ± 0.4	6.7 ± 0.1	0.042 ± 0.004	0.628 ± 0.058	0.076 ± 0.020

Fisher's least significant difference test. A difference of $P < 0.05$ was considered significant. Values are presented as means ± SD.

RESULTS

Targeted disruption of the SCD1 gene. Figure 1A[2] shows the strategy that was used to disrupt the SCD1 gene. The mutant mice were viable and fertile, and bred with predicted Mendelian distributions. PCR of genomic DNA using the designated primers showed a band of 425 bp (Fig. 1B) that would be expected in the SCD1−/− mice. The SCD1+/− DNA showed both the 425- and 600-bp fragments, whereas the wild-type DNA showed the expected 600-bp fragment. To determine whether the expression of the SCD1 gene was ablated, we performed Northern blot analysis of mRNA isolated from several tissues (Fig. 1C). SCD1 mRNA was expressed in liver, eyelid, white adipose tissue (WAT) and skin of wild-type mice, reduced by ~50% in SCD1+/− mice and undetectable in the SCD1−/− mice. SCD2 mRNA was expressed at similar levels in the eyelid, WAT, skin, brain and eyeball of both SCD1−/− mice and wild-type mice but undetectable in the liver, consistent with published results (2). SCD enzyme activity in liver, as measured by the rate of conversion of [1-^{14}C]stearoyl-CoA to [1-^{14}C]oleate (Fig. 1D), was high in the wild-type mice, intermediate in the SCD1+/− and undetectable in the total extracts of livers of the SCD1−/− mice. Consistent with the Northern blot results, Western blot analysis showed high immunoreactive SCD protein in liver from the wild-type, intermediate in the SCD1+/− and none in the SCD1−/− mice (Fig. 1D). Table 1 shows the fatty acid composition of liver, eyelid, WAT, skin, brain and eyeball. The relative amount of palmitoleate [16:1(n-7)] in liver from SCD1−/− mice was 55% lower ($P < 0.05$) than in wild-type mice, whereas that of oleate [18:1(n-9)] was 35% lower ($P < 0.05$). The relative levels of palmitoleate in WAT and skin of the SCD1−/− mice were >70% lower than in wild-type mice (WAT, $P < 0.01$; skin, $P < 0.01$), whereas the decrease in the level of oleate in these tissues was ~20% (WAT, $P < 0.001$; skin, $P < 0.05$). These changes in the levels of MUFA resulted in greater levels of the corresponding saturated fatty acids (18:0 and 16:0) and lower desaturation indices, indicating a reduction in desaturase activity. The relative levels of 16:1(n-7) and 18:1(n-9) in liver, eyelid, WAT and skin of SCD+/− mice were between those of wild-type and SCD1−/− mice. In contrast to the liver, WAT and skin, the brain, which expresses predominantly the SCD2 isoform, had a similar fatty acid composition and unaltered desaturation index both in the wild-type and SCD1−/− mice. We conclude that our disruption resulted in mice null for the SCD1 gene and that the expression of SCD2 isoform does not compensate for SCD1 deficiency.

Gross pathology and histological examination of SCD1−/− mice. The SCD1−/− mice exhibited cutaneous abnormalities and a narrow eye fissure, which started around the weaning age (3–4 wk). The abnormalities became more severe with aging. The SCD1−/− mice had a thinner hair coat than the wild-type control mice. The hair loss in the SCD1−/− mice was apparent throughout the whole skin area. Histological examination of the skin of the SCD1−/− mice revealed atrophy of the sebaceous glands, whereas the wild-type mice had prominent and well-differentiated sebaceous glands (compare Fig. 2A for wild-type with Fig. 2B for SCD1−/−). Figure 2C is a higher magnification of the sebaceous gland of a wild-type mouse, showing a foamy appearance of the cytoplasm due to the presence of lipid droplets, the

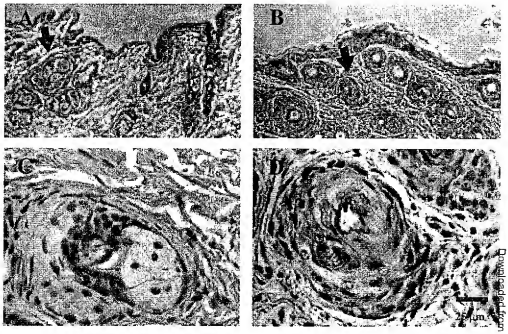

FIGURE 2 Histology of skin of wild-type and SCD1−/− mice. The mice were 2-mo-old males. (*A*) Hematoxylin and eosin (H&E) staining of a wild-type mouse shows normal epidermal structure with a well-differentiated sebaceous gland (arrowhead). (*B*) H&E staining of skin of a SCD1−/− mouse shows atrophic sebaceous gland (arrowhead). (*C*) Higher magnification (×400) of the sebaceous gland of the wild-type mouse. Note the foamy appearance of the cytoplasm. (*D*) Higher magnification (×400) of the sebaceous gland of the SCD1−/− mouse showing atrophic sebaceous gland. The foamy appearance of the cytoplasm seen in the wild type has disappeared.

sebum. In contrast, at a higher magnification, the cytoplasm of the atrophic sebaceous gland in the SCD1−/− mouse had lost the foamy appearance (Fig. 2*D*), suggesting depletion of sebum lipids. The SCD1−/− mice had narrower eye fissures than wild-type mice. Histological examination of the eyelid of the SCD1−/− mice revealed atrophy of the meibomian gland, a specialized sebaceous gland (compare **Fig.** 3B for the SCD1−/− mice with 3A for wild type) that secretes meibum, the oily material that prevents the corneal surface of the eye from drying out. At a higher magnification (Fig. 3C), the cytoplasm of the meibomian gland of the wild-type control mouse appears foamy due to the presence of meibum, whereas the meibomian gland of the SCD1−/− mouse lacks the foamy appearance (Fig. 3*D*) due to depletion of the meibum lipids. No abnormalities were found in cornea and retina of the SCD1−/− mice (data not shown).

The skin and eyelid of SCD1−/− mice are deficient in cholesterol esters, wax esters and triglycerides. We measured free cholesterol, cholesterol ester, triglycerides and wax ester content in the skin and eyelid. HPTLC of lipids extracted from the skin of wild-type mice showed that the skin contained very high levels of triglycerides (Fig. 4A), whereas the eyelid was very rich in wax esters (Fig. 4C). The triglycerides, cholesterol ester, and wax ester levels were markedly

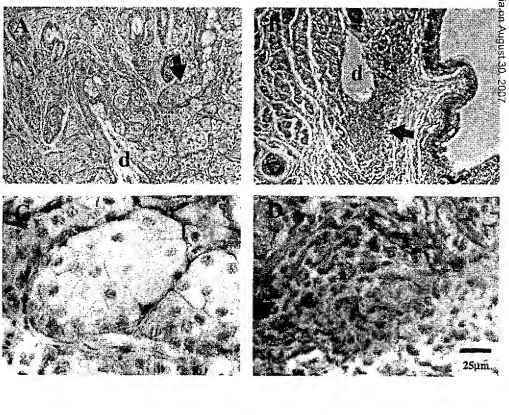

FIGURE 3 Histology of the eyelid of wild-type and SCD1−/− mice. The mice were 2-mo-old males. (*A*) Hematoxylin and eosin (H&E) staining of a wild-type mouse shows a normal well-differentiated meibomian gland (arrowhead). Intralobular duct (d). (*B*) H&E staining of the eyelid of a SCD1−/− mouse shows atrophic meibomian glands (arrowhead). (*C*) Higher magnification (×400) of the meibomian gland of the wild-type mouse. Note the foamy appearance of the cytoplasm. (*D*) Higher magnification (×400) of the atrophic meibomian gland of the SCD1−/− mouse. The foamy appearance observed in the wild-type meibomian gland has disappeared.

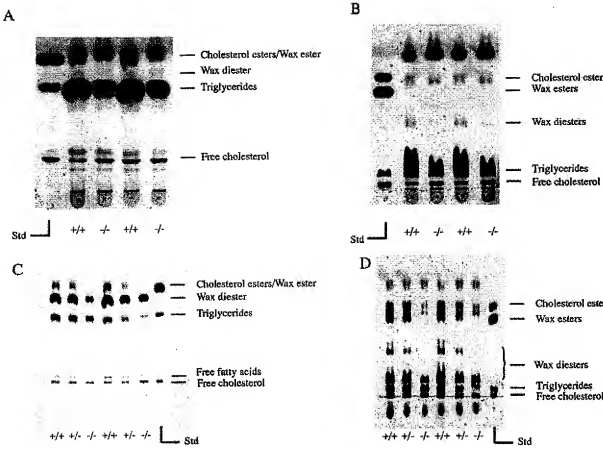

FIGURE 4 High performance TLC (HPTLC) of lipids extracts from skin (*A* and *B*) and eyelids (*C* and *D*) of wild-type and SCD1−/− mice. Total lipid extracts were pooled and analyzed by HPTLC. Equivalent amounts of lipid extract (from 0.5 mg of eyelid or skin) were loaded in each lane. Each lane represents lipids from eyelid and skin samples of two mice. In *A* and *C*, the solvent system was hexane/ether/acetic acid (90:25:1). In *B* and *D*, the solvent system used to resolve the wax esters was benzene/hexane (65:35). Std, standards.

reduced in both the skin and eyelid of the SCD1−/− mice compared with the wild-type control mice. Intermediate levels were observed in the lipids of the heterozygote mice (SCD1+/−). A separate TLC analysis using a solvent system consisting of hexane/benzene (45:65, v/v) was performed to separate (based on R_f values) cholesterol esters from wax esters and also to resolve the wax diesters and triesters (15). As shown, the diester was the major wax ester in the eyelid (Fig. 4*D*). The skin also contained wax diesters (Fig. 4*B*) but at much lower levels than the eyelid diesters. The cholesterol ester concentrations in eyelid and skin of SCD1−/− mice were 73% ($P < 0.001$) and 43% ($P < 0.05$) lower, respectively, than in wild-type mice, whereas free cholesterol levels in skin and eyelid were 57% ($P < 0.001$) and 93% ($P < 0.001$) greater, respectively (Table 2). The total wax ester concentration in the eyelid of the SCD1−/− mice compared with the wild-type mice was 72% lower ($P < 0.001$). The total wax ester concentration of the skin was too low for accurate measurement. The triglyceride concentrations in the skin and eyelid were 53% ($P < 0.001$) and 60% ($P < 0.001$) lower, respectively, than in wild-type mice.

In the SCD1−/− mice, the concentrations of MUFA including18:1(n-9) ($P < 0.01$), 18:1(n-7) ($P < 0.01$) and 16:1(n-7) ($P < 0.01$) in the total lipid fraction were >55% lower than in wild-type mice (Figure 5A). In the cholesterol ester fraction, the levels of 18:1(n-9) ($P < 0.001$) and 16:1(n-7) ($P < 0.01$) were reduced by >70%. The triglyceride fraction of the wild-type control mice contained high levels of 16:1(n-7) and 18:1(n-9), which were reduced by 51 and 71% ($P < 0.01$), respectively, in the SCD−/− mice. In the wax ester fraction, the levels of 18:1(n-9) ($P < 0.01$), 18:1(n-7) ($P < 0.01$) and 16:1(n-7) ($P < 0.01$) were >50% lower than in wild-type mice.

TABLE 2

Eyelid and skin lipids in wild-type (+/+) and SCD homozygous (−/−) mice fed a semipurified diet[1]

Genotype	+/+	−/−
	μmol/g tissue	
Eyelid		
Cholesterol ester	27.8 ± 1.1	7.4 ± 0.5*
Free cholesterol	13.8 ± 1.3	21.7 ± 0.5*
Wax ester	69.4 ± 8.3	19.4 ± 1.5*
Triglyceride	15.6 ± 0.7	6.2 ± 0.5*
Phospholipid	7.7 ± 0.3	7.7 ± 0.4
Skin		
Cholesterol ester	0.7 ± 0.1	0.4 ± 0.2**
Free cholesterol	5.9 ± 0.5	11.4 ± 1.0*
Triglyceride	87.3 ± 0.3	41.5 ± 7.6*

[1] Values are means ± SD, $n = 4$. All mice were 6-wk-old males.
* Significantly different ($P < 0.001$) from wild-type mice (Student's *t* test).
** Significantly different ($P < 0.05$) from wild-type mice (Student's *t* test).

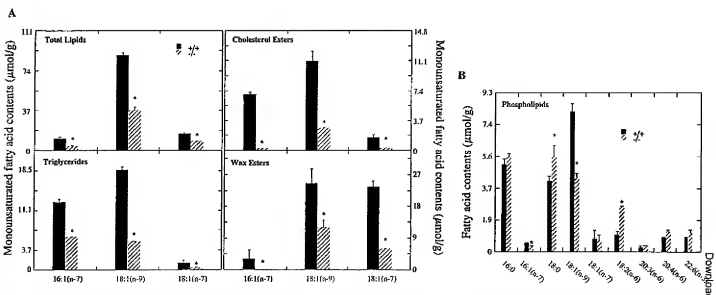

FIGURE 5 The concentrations of monounsaturated fatty acids in the total lipid fraction, the cholesterol ester, wax ester, and triglyceride fractions (*A*) and in the phospholipid fraction (*B*) of eyelid of wild-type and SCD1−/− mice. Lipid extracts in (*A*) were pooled and analyzed by TLC, methyl esterified and quantitated by gas-liquid chromatography (GLC). Values are means ± sd, $n = 3$. *Significantly different ($P < 0.05$) from wild-type mice.

Figure 5B shows the concentrations (μmol/g) of the major fatty acids measured in the phospholipid fraction. Although there was no alteration in the molar amount of the phospholipid pool (data not shown), differences in the acyl chain concentrations were observed. The levels of 18:1(n-9) and 16:1 were 48% ($P < 0.01$) and 33% ($P < 0.05$) lower, respectively, in SCD−/− mice than in wild-type mice. The levels of 18:0 were greater ($P < 0.01$) than in wild-type mice. There was also a >50% greater ($P < 0.05$) concentration of 18:2(n-6) in the SCD−/− mice. The quantitative data on the composition of the acyl chains indicated that although the eyelid triglyceride, cholesterol ester and wax ester pools were reduced (Fig. 4), the total MUFA concentration in each fraction was dramatically reduced in SCD1−/− mice. In addition, the changes observed in the fatty acid content of the phopsholipid pool indicate that SCD1 deficiency alters the acyl chain composition of membrane phospholipids.

High levels of dietary 18:1(n-9) did not alter triglyceride, cholesterol ester and wax ester levels in the eyelid of SCD1−/− mice. The cellular oleate or palmitioleate used for cholesterol ester, triglyceride and wax ester synthesis in mouse eyelid can be synthesized either de novo or by desaturation of exogenous stearate or palmitate derived from the diet. To determine whether dietary oleate could substitute for the endogenously synthesized oleate and restore the levels of triglycerides, esters and wax esters to the levels found in the eyelid of the wild-type mouse control, we supplemented the semipurified mouse diet with high levels of oleate as triolein. This diet was then fed to the SCD1−/− mice for 2 wk, long enough to ensure equilibration of lipid pools. Total eyelid extracts were prepared; the lipid fractions were analyzed by TLC and the fatty acid composition was analyzed by GLC. Feeding diets supplemented with triolein to the SCD1−/− mice did not increase the levels of triglycerides, cholesterol esters or wax esters (Fig. 6*A*). GLC analysis also showed that the levels of 18:1(n-9) were not increased in the total lipid fraction of the eyelid (Fig. 6*B*). The mice maintained the eye fissures and the thin hair coat phenotype of the SCD1−/− mice that consumed the semipurified diet. No changes in phenotype or in levels of neutral lipids were observed when the SCD1−/− mice were fed diets rich in 16:1 or a combination of 18:1 and 16:1 (data not shown). These results suggest that the endogenously synthesized 18:1 or 16:1 by SCD constitute a pool of MUFA required for the synthesis of the eyelid or skin cholesterol esters and triglycerides and further confirm the requirement for these fatty acids in the synthesis of eyelid wax diesters.

DISCUSSION

The mouse genome contains three well-characterized structural genes (SCD1, SCD2 and SCD3) that are highly homologous at the nucleotide and amino acid levels and encode the same functional protein. Although the difference in physiologic function among the three SCD isoforms has not been well addressed, we previously suggested that the SCD isoforms might exhibit different specificity for substrates. For example, SCD1 is relatively nonselective and uses both stearoyl-CoA (18:0-CoA) and palmitoyl-CoA (16:0-CoA) as substrates, whereas SCD2 is specific for stearoyl-CoA as a substrate (10). Palmitate and stearate can be synthesized de novo or can be derived from the diet. Oleate and palmitoleate, which are the corresponding MUFA, then serve as the major fatty acid substrates for incorporation into membrane phospholipids, cholesterol esters and triglycerides. In addition to being components of these major lipids, MUFA have also been implicated as mediators of several physiologic processes including signal transduction and cellular differentiation (10,20). Some clues to the physiologic role of the SCD1 gene and its endogenous products (MUFA) have been obtained by characterizing the phenotypes of two naturally occurring mouse models (ab^J and ab^{2J}) carrying mutations in the SCD1 (5,11,12). The homozygous ab^{2J} SCD1 mutant mouse is similar to the ab^J mouse except for the extent of epidermal thickness, scaling and epidermal permeability barrier (12). The basis for the difference between the two alleles with a mutation in the same gene is not yet understood but could be due to differences in the backgrounds of the two mouse strains.

To further explore the physiologic roles of the SCD gene isoforms and to reveal other phenotypes not previously char-

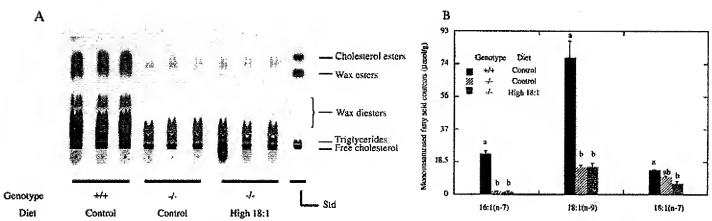

FIGURE 6 Lipids and monounsaturated fatty acid concentrations in eyelids of wild-type and SCD1−/− mice fed high oleate diets. At 6 wk of age, the mice were fed a control (5 g soybean oil/100 g) diet or high oleate (5 g 18:1/100 g) diet for 2 wk. (*Panel A*) Total lipids were extracted from eyelids of wild-type and SCD1−/− mice. Lipid extracts were analyzed by high performance TLC (HPTLC). Equivalent amounts of lipid extract (from 0.5 mg eyelid) were loaded in each lane. Each lane represents lipids from eyelid of one mouse. Std; standards. (*Panel B*) Total lipids were extracted from 2 mg of harderian gland. The monounsaturated fatty acid contents were quantified by gas-liquid chromatography. Values are means ± SD, $n = 3$. Means with a different superscript letter are significantly different ($P < 0.05$).

acterized in the ab^{J} and ab^{J2} mice, we generated mice with a targeted disruption of the SCD1 gene. Like the ab^{J} and ab^{J2}, the SCD1−/− mice exhibited cutaneous abnormalities with atrophic sebaceous gland and narrow eye fissure with atrophic meibomian glands. However, unlike the asebia (ab^{J}) and ab^{J2}, which have reduced levels of cholesterol in their skin, the skin and eyelid of our SCD1−/− mice had significantly greater levels of free cholesterol than the wild-type mice. The SCD−/− mice, like the ab^{J} mice, had decreased concentrations of liver cholesterol and triglyceride, as well as plasma VLDL-triglycerides (data not shown). However, differences were noted in the levels of lipoproteins compared with the previously reported plasma lipoprotein profile of ab^{J} mice (4) and these will be reported elsewhere. These differences could also be due to mouse strain backgrounds modifying gene effects. In this study, a more extensive analysis of the changes in lipid and fatty acid composition of several tissues was carried out. Figures 4–6 and Table 2 show that the SCD1−/− mice were deficient in eyelid triglycerides, cholesterol esters and wax esters. These deficiencies in the lipid pools can be attributed to decreases in the endogenous levels of 18:1 and 16:1. The increase in the levels of 18:2(n-6) observed in the phospholipid fraction (Fig. 5B) indicates that SCD1 deficiency alters the acyl chain composition of membrane phospholipids.

The cutaneous abnormalities in the SCD1−/− mice as well as those observed in the ab^{J} and ab^{J2} mice are similar to those reported in transgenic mice overexpressing human apolipoprotein (apo)CI (21) and in mice deficient in acyl-CoA:cholesterol acyl transferase enzyme (ACAT-1) (19). ApoC1 activates lipoprotein lipase to facilitate VLDL clearance from plasma, but an excess of apoC1 has been shown to impair hepatic uptake of VLDL (21). Like the SCD−/− mice (Figs. 2, 3), apoCI mice exhibit atrophic sebaceous and meibomian glands as well as a decrease in wax diesrer and triglycerides of the epidermis. The skin of mice overexpressing apoC1 also has increased levels of free cholesterol but unlike our SCD1−/− mice, the cholesterol ester levels in these tissues are not altered. Like the SCD−/− mice, the ACAT-1–deficient mice are deficient in cholesterol ester and have more free cholesterol in the eyelid with atrophy of the meibomian glands. The deficiency in cholesterol ester of the SCD1−/− mice is most likely due to lack of MUFA produced by SCD as substrates of ACAT, but the relationship between endogenously synthesized MUFA and the phenotype of mice with high expression of apoC1 is less clear. However, the phenotypic feature that the SCD1−/−, ACAT-1−/− and mice overexpressing human apoCI have in common is the increased levels of free cholesterol either in the eyelid or skin or both. Because excess free cholesterol can lead to cell death (22), it is tempting to speculate that atrophy of the sebaceous and meibomian glands may be due to an increase in the amount of cellular free cholesterol in these glands rather than the reduced levels of sebum and meibum.

We hypothesized that feeding high levels of dietary oleate and palmitoleate could correct the deficiency in triglyceride, cholesterol ester and wax ester in the eyelids of SCD1−/− mice. However, upon supplementing the semipurified diets with high levels of oleate as triolein and feeding this diet to the SCD1−/− mice for 2 wk, we found no increase in the levels of triglycerides, cholesterol esters or wax esters (Fig. 6A). GLC analysis also showed that the levels of 18:1(n-9) were not increased in the total lipid fraction of the eyelid (Fig. 6B). Dietary oleate could not increase the levels of wax diester, cholesterol ester and triglyceride in the skin of the SCD1−/− mice (data not shown). No changes in phenotype were observed when the SCD1−/− mice were fed diets rich in 16:1 or a combination of 18:1 and 16:1. These feeding experiments argue against the idea that dietary intake is ultimately the major lipid source of the MUFA that are incorporated into cellular neutral lipids. The present data are consistent with our previous concept (4) that endogenously synthesized oleate and palmitoleate, which arise from SCD activity in the endoplasmic reticulum, provide the bulk of the monounsaturated fatty acid pool and are preferred substrates for the enzymes of triglyceride, cholesterol and wax ester biosynthesis. It is possible that dietary MUFA and the SCD-synthesized MUFA are functionally compartmentalized into distinct lipid pools, thereby suggesting a mechanism of neutral lipid biosynthesis that is unknown at this time. The deficiencies in the neutral lipids exist despite the expression of the SCD2 gene in the eyelid of SCD1−/− mice (Fig. 1C), suggesting that SCD2 cannot compensate for the SCD1 deficiency in this tissue.

It was reported recently that chronic blepharitis and dry eye syndrome, which constiture one of the most common and frustrating eye disease conditions in humans, are due to lipid abnormalities in meibum (23–26), but the nature of these lipid

abnormalities has not been well characterized. It has been found, however, that meibum from patients with meibomian keratoconjunctivitis has decreased levels of oleic acid, a major product of SCD, whereas that from patients with meibomian seborrhea has increased levels of oleic acid (26). These observations, together with our present study, suggest that the alteration of SCD activity in the eyelid can be implicated in human eye diseases. Meibum is a mixture of triglycerides, cholesterol esters and wax esters. It is possible that these neutral lipids synergistically endow this semiliquid mixture with the physical properties, such as correct viscosity and melting point, necessary to prevent the corneal surface of the eye from drying out by retarding evaporation of tear moisture. Thus, the deficiencies in meibum, together with the dysfunction of meibomian glands, are most likely the cause of the narrow eye shape or dry eye syndrome observed in SCD1−/− mice and humans, respectively. The eyelids of SCD1−/− mice deficient in meibum have more frequent bacterial infections than those of wild-type control mice, suggesting that eyelid meibum may be toxic to some pathogenic microorganisms, although this property remains to be established.

In conclusion, the characterization of the targeted SCD knockout mouse we have generated allows another experimental model, in addition to the naturally occurring models of SCD1 disruption, with which to study lipid metabolism in both normal and disease states. The studies reported here provide substantial evidence that SCD1 gene expression plays a major role in triglyceride, cholesterol ester and wax ester biosynthesis in the skin and eyelid. The enzymes that synthesize triglycerides, cholesterol esters and wax esters require 18:1 and 16:1, the endogenous products of SCD, as substrates. Thus, the SCD1 gene may be a major checkpoint in the processes of cholesterol homeostasis, lipoprotein and neutral lipid metabolism. The ocular complications exhibited by the SCD1 knockout mouse and the failure of high levels of dietary MUFA to repair these defects strongly suggest differences in the metabolic functions of de novo synthesized and dietary MUFA. The studies described here may have broad implications for potential use of the SCD1 gene as a target in the treatment of human eye and skin diseases.

ACKNOWLEDGMENTS

We thank Juris Ozols (University of Connecticut Health Center, Farmington, CT) for the antibody to the rat liver microsomal stearoyl-CoA desaturase. We thank Alan Attie for useful discussions.

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Lipid Level and Type Alter Stearoyl CoA Desaturase mRNA Abundance Differently in Mice with Distinct Susceptibilities to Diet-Influenced Diseases[1,2]

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ABSTRACT Chronic diseases develop in susceptible individuals following exposure to environmental conditions including high fat diets. Inbred strains of mice differing in susceptibility to atherosclerosis, diabetes, obesity and certain cancers are models for understanding the genetic basis and molecular mechanisms whereby diet influences these polygenic and multifactorial disorders. Expression sequence tags (EST) and disease quantitative trait loci (QTL) are also being identified with these strains. Reported here are comparisons of food intake, growth, nonfasting serum lipids and expression of mRNA for hepatic apolipoprotein E (ApoE), hepatic stearoyl CoA desaturase (Scd1) and heart lipoprotein lipase (Lpl) in a 2 × 2 × 2 design with C57BL/6J and BALB/cByJ mice fed semipurified diets with 4 or 20% saturated (coconut) or unsaturated (corn) oils for 4 mo. Histological studies of aortas and coronary arteries are also reported for these animals. After 4 mo, BALB/cByJ mice were significantly heavier and had significantly higher total serum cholesterol, HDL cholesterol and triglyceride concentrations in the fed state than C57BL/6J mice. Efficiency of utilizing dietary energy did not differ consistently between strains. Oil level affected serum total cholesterol, triglycerides and HDL cholesterol, which were significantly greater in mice fed high fat diets. Lpl and ApoE mRNA expression levels were not significantly affected by mouse strain, oil source or oil level. Scd1 mRNA expression, however, was significantly higher in C57BL/6J than in BALB/cByJ mice and was lower in all mice fed 20% compared with those fed 4% fat diets. Genes regulated differently by diet among strains with distinct susceptibility to diet-influenced disease may be associated with molecular pathways contributing to incidence or severity. J. Nutr. 127: 566–573, 1997.

KEY WORDS: • *diet* • *inbred mouse strains* • *serum lipids* • *stearoyl CoA desaturase* • *disease genes*

Epidemiological and laboratory animal studies indicate that diets high in fat increase the incidence and severity of atherosclerosis, diabetes, obesity and cancer in susceptible individuals (reviewed in NRC 1989). Because diet changes disease phenotype, certain dietary components must regulate expression of a subset of genes whose involvement in disease development (Berg 1989, Kaput et al. 1994, Kirk et al. 1995) appears to be regulated differently in genetically distinct individuals. Except for familial and dominant mutations, chronic diseases are outcomes of contributions from many genes interacting with environmental factors (e.g., Berg 1989, Grundy 1995, Hegele 1992, Kirk et al. 1995, NRC 1989).

Certain inbred strains of mice, established by at least 20 generations of brother × sister matings, show higher susceptibility than other strains to experimentally induced disease. They have been used experimentally to identify quantitative and qualitative chromosomal loci (reviewed in Frankel 1995) and to define molecular events responsible for disease development. Changes in abundances of mRNA-encoding genes participating in lipid metabolism have also been compared within or between inbred mouse strains fed normal diets (Kirk et al. 1995, Srivastava 1996, Srivastava et al. 1991 and 1992) or atherosclerosis-inducing diets (Kirchgessner et al. 1989, LeBeouf et al. 1994, Liao et al. 1993, Qiao et al. 1993, Uelmen et al. 1995, Warden et al. 1989). Based upon the evidence, some diet-regulated or other genes are likely to participate in disease induction or severity. These have been referred to as level and variability genes (Berg 1989, Kirk et al. 1995).

Our laboratories have collaborated in developing a

[1] Supported by grants from the U.S. Department of Agriculture (91-01631), The American Institute for Cancer Research (NC893B63), a gift from Abbot Laboratories (Columbus, OH) and the University of Illinois Colleges of Medicine and Agriculture. D.A.S. and E.A.P. were supported by predoctoral fellowships from the American Institute of Nutrition. D.A.S. was also supported by a National Institutes of Health traineeship (PHS ST-92AM07497).

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0022-3166/97 $3.00 © 1997 American Society for Nutritional Sciences.
Manuscript received 7 August 1996. Initial review completed 23 September 1996. Revision accepted 10 January 1997.

multistep procedure (Elliott et al. 1993, Kaput et al. 1994, Paisley et al. 1996, Swartz et al. 1996) for identifying diet-regulated genes and for testing the hypothesis that such genes participate in disease development (Kaput et al. 1994 and this report). The first step in our protocol involves the isolation of genes or expression sequence tags (EST)[7] regulated by diet (Elliott et al. 1993) in tissues of disease-free virgin female mice fed semipurified diets. When desirable, the precision of the model can be refined by the feeding of chemically purified diets. Step 2 analyzes mRNA abundance between inbred strains differing in disease susceptibility before signs of the disease are evident. Our working hypothesis is that genes regulated differently between strains by the same diet may be among the subset involved in producing differences in disease phenotypes between strains. The third step compares the chromosomal map position of the differently regulated genes to independently derived quantitative trait loci (QTL). Others have proposed mapping disease-specific expressed sequence tags (EST) with independently derived QTL (Berry et al. 1995) for identifying candidate disease genes, an example of association analyses (Risch and Merikangas 1996). EST regulated by the same nutritional factors that produce the disease and overlapping disease QTL maps have been defined as candidate disease genes (Risch and Merikangas 1996) for analyses in humans or animals showing the disease, the last step of our proposed protocol.

We previously isolated hepatic apolipoprotein E (ApoE) and stearoyl CoA desaturase (Scd1) (Elliott et al. 1993) using the above described experimental model in screens for diet-regulated genes. We report here the analyses of hepatic ApoE, hepatic Scd1, and heart Lpl mRNA abundance in C57BL/6J and BALB/cByJ mice fed semipurified diets with 4 or 20% corn or coconut oils for 4 mo. C57BL/6 are more susceptible to diet-induced atherosclerosis (Paigen et al. 1987 and 1990), type II diabetes (Seldin et al. 1994, Surwit et al. 1995), and express certain genes involved in lipid metabolism differently than BALB/c mice (Kirk et al. 1995 and this report). Food intake, growth, nonfasting serum lipids, histological sections of aortas and coronary arteries were also analyzed to assess their influence or correlation with gene regulation. This report is an example of the second step of our protocol.

MATERIALS AND METHODS

Animals, diets and protocols. Eighty virgin female BALB/cByJ and C57BL/6J mice, 6–7 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). They were fed a semipurified diet containing 4% corn oil for 1 wk and then randomly assigned to diets containing 4% corn oil, 20% corn oil, 4% coconut oil or 20% coconut oil for an additional 15 wk (Fig. 1). Each diet contained 1.4% of the respective total oil content as soybean oil to assure adequate fatty acid content (NRC 1995, Reeves et al. 1993). The diets (Table 1), formulated according to modified AIN-76 guidelines (NRC 1995, Reeves et al. 1993), were pelleted and color coded by Research Diets (New Brunswick, NJ). Mice were caged and fed individually with free access to food and distilled water in temperature-controlled rooms maintained at 23 ± 1°C with a 12-h light:dark cycle. Animal care met University of Illinois and National Institutes of Health guidelines. Food spillage was also monitored throughout the course of the experiment. Efficiencies of energy utilization were calculated from the recorded weekly weight gain/calculated weekly energy intake. At 16 wk, all mice were deprived of food for 12 h and offered a pre-weighed 3-g pellet of their assigned diet. After 2 h, the uneaten food was removed; 2 h later, all mice were injected intramuscularly with 0.02 mL/g body weight of ketaset/xylazine mixture (Ketaset, Fort Dodge Laboratories, Ft. Dodge, IA) for collection of blood via cardiac puncture. Immediately thereafter, they were killed by cervical dislocation and their livers and hearts were removed, individually frozen in liquid nitrogen, and stored at −80°C for mRNA isolation.

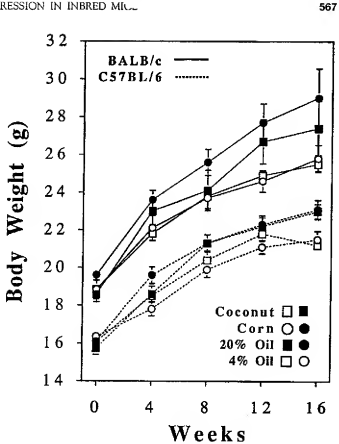


FIGURE 1 Body weights of C57BL/6J and BALB/cByJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. Body weights (means ± SEM) and food intakes (see text) for five C57BL/6J and five BLB/cByJ mice fed each dietary treatment were monitored twice per week during the feeding study and the averages within each group at monthly intervals are plotted. BALB/cByJ mice at 6–7 wk of age weighed more initially than C57BL/6J mice (19.0 vs. 16.1 g, respectively) at 6–7 wk of age and throughout the 16-wk feeding study ($P < 0.0001$). BALB/cByJ mice averaged 26.9 g compared with 22.2 g for C57BL/6 mice at the end of the study.

DNA probes. cDNAs for lipoprotein lipase (Genbank/EMBL: J02740), rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mouse β-actin and stearoyl CoA desaturase (Scd1, Genbank/EMBL: J04190, M21285) were as described previously (Paisley et al. 1996, Swartz et al. 1996). A DNA fragment encoding nucleotides 681 to 806 of apolipoprotein E mRNA (Genbank/EMBL: D00466, M12414) was isolated in a second screen for diet-regulated genes. cDNA were excised from the vectors, isolated (Ausubel et al. 1987) and labeled by random priming or polymerase chain reaction (PCR) amplification in the presence of α-^{32}P-deoxycytidine-5-triphosphate (dCTP, 111 TBq/mmol, ICN, Irvine, CA) as described by Paisley at al. (1996) and Swartz et al. (1996).

Northern hybridization analyses. Twenty micrograms of total RNA, isolated from livers by the Ultra-spec II RNA isolation system (Biotecx, Houston, TX), was resolved by electrophoresis in gels containing 1.2% agarose and 2.2 mol/L formaldehyde for 2.5 h at 110 V. Sizes of mRNA were estimated by comparisons to a synthetic RNA ladder electrophoresed simultaneously. Gels were subsequently stained with 0.5 mg/L ethidium bromide and photographed to verify rRNA quality. RNA was blotted onto nylon membranes (U.S. Biochemical, Cleveland, OH) following standard protocols (Ausubel et al. 1987). Blots were prehybridized at 42°C with 50% formamide, 5X

[7] Abbreviations used: ApoE, apolipoprotein E; Chr, chromosome; EST, expressed sequence tags; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Lpl, lipoprotein lipase; PCR, polymerase chain reaction; QTL, quantitative trait loci; Scd1, stearoyl CoA desaturase; SSC, standard sodium citrate.

TABLE 1

Diet composition

Ingredient	Corn		Coconut	
	4%	20%	4%	20%
	g			
Corn oil	2.8	18.6	0	0
Coconut oil	0	0	2.8	18.6
Soybean oil	1.4	1.4	1.4	1.4
Maltodextrin	11.8	14.1	11.8	14.1
Cornstarch	45.0	19.3	45.0	19.3
Sucrose	9.5	11.2	9.5	11.2
Casein, alcohol extracted	19.0	22.6	19.0	22.6
L-Cystine	0.3	0.3	0.3	0.3
Cellulose	4.7	5.6	4.7	5.6
Mineral mix[1]	1.0	1.1	1.0	1.1
Calcium phosphate	1.2	1.5	1.2	1.5
Calcium carbonate	0.6	0.7	0.6	0.7
Potassium citrate, monohydrate	1.6	1.9	1.6	1.9
Vitamin mix[1]	1.0	1.1	1.0	1.1
Choline bitartrate	0.2	0.2	0.2	0.2
Total grams	100.1	94.2	100.1	94.2
kJ/g[2]	15.9	19.2	15.9	19.2
Protein, kJ%	20.0	20.0	20.0	20.0
Lipid, kJ%	10.0	40.0	10.0	40.0
Carbohydrate, kJ%	70.0	40.0	70.0	40.0
Minerals, mg/kJ	2.7	2.6	2.7	2.6
Vitamins, mg/kJ	0.6	0.6	0.6	0.6
Cellulose, mg/kJ	3.0	2.9	3.0	2.9

1 AIN-76A diet recommendations (National Research Council 1995).
2 Amount of energy is 16.7 kJ/g for protein and carbohydrate, 37.6 kJ/g fat.

standard sodium citrate (SSC), 1X Denhardt's solution, 0.02 mol/L sodium phosphate (pH 6.8), 0.1 g/L denatured salmon sperm DNA, 1 mmol/L EDTA, 0.2% SDS and 10% dextran sulfate (Ausubel et al. 1987). After overnight hybridization at 42°C, the membranes were washed twice at room temperature with 1X SSC containing 0.5% SDS for 20 min. Two 15-min washes with 0.5X SSC/0.5% SDS and one 15-min wash in 0.25X SSC/0.5% SDS at 65°C followed. Membranes were placed next to preflashed (Laskey 1980) Kodak XAR

TABLE 2

Serum total cholesterol, HDL cholesterol and triglyceride concentrations in BALB/cByJ and C57BL/6J mice fed 4 or 20% corn oil or coconut oil for 15 wk[1,2]

	Serum total cholesterol	Triglyceride	HDL cholesterol
		mmol/L	
Mouse strain			
BALB/cByJ	1.64 ± 0.04***	0.94 ± 0.06*	1.07 ± 0.06***
C57BL/6J	1.36 ± 0.05***	0.76 ± 0.06*	0.73 ± 0.05***
Level of oil			
4%	1.40 ± 0.05**	0.73 ± 0.04**	0.81 ± 0.05**
20%	1.59 ± 0.05**	0.97 ± 0.07**	0.98 ± 0.07**
Source of oil			
Corn	1.47 ± 0.05	0.80 ± 0.07	0.92 ± 0.07
Coconut	1.52 ± 0.05	0.89 ± 0.05	0.86 ± 0.06

1 Values are means ± SEM (n = 29–39).
2 Significant differences are * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. Main effect differences in total cholesterol, triglycerides, or HDL are between mouse strains (BALB/cBYJ vs. C57BL/6J) or between levels of oil (4% vs. 20%).

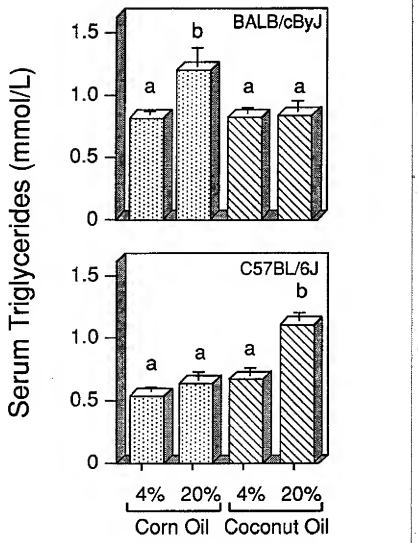


FIGURE 2 Serum triglyceride concentrations in C57BL/6J and BALB/cByJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. Serum triglyceride concentrations (means ± SEM) are reported for 8–10 C57BL/6J and 8–10 BALB/cByJ mice fed each dietary treatment (except BALB/cByJ fed 20% coconut oil, where n = 5). Triglyceride concentrations were affected by interactions between strain and source ($P < 0.0009$) and among strain, oil source and oil level ($P < 0.014$). The latter interaction is indicated by (a,b) where values with the same letter differ from values with different letters. See Table 2 for main effects.

film at −80°C, and electrophoretic patterns were analyzed in the linear range of the film (Laskey 1980). Membranes of liver RNAs were rehybridized with a β-actin to control for the total RNA per well. Glyceraldehyde-3-phosophate dehydrogenase (Paisley et al. 1996) served as the control for RNA in hearts, which express two actin mRNAs. β-Actin and GAPDH are believed to be unaffected by changes in metabolism and are used as controls for mRNA abundance measurements (e.g., Ausubel et al. 1987). Because ethidium bromide staining of nucleic acids is a relatively insensitive measure of RNA loading with 10–20 μg loaded per lane, mRNA abundance is reported as the ratio of specific transcripts to control transcripts. RNA from livers of 80 mice (10 from each dietary treatment) and hearts from 40 mice (5 from each dietary treatment) were analyzed. Hybridization signals were quantified with a Molecular Dynamics 425S phosphorimager (Molecular Dynamics, Sunnyvale, CA). Abundances of specific mRNA were normalized to the amount of β-actin or GAPDH mRNA within the same electrophoretic lane.

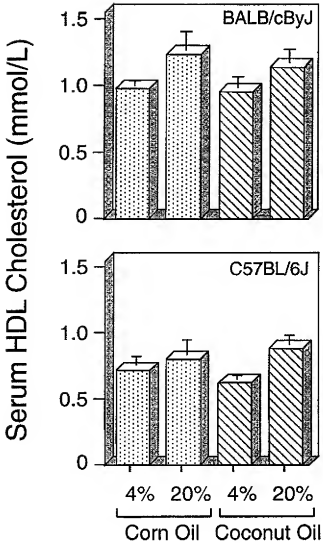


FIGURE 3 HDL cholesterol concentrations in C57BL/6J and BALB/cByJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. HDL cholesterol concentrations (means ± SEM) are reported for 8–10 C57BL/6J and 8–10 BALB/cByJ mice fed each dietary treatment (except BALB/cByJ fed 20% coconut oil, where $n = 6$, and C57BL/6J mice fed 4% corn oil, where $n = 7$). There were no significant interactions for HDL cholesterol concentrations. See Table 2 for main effects.

Serum lipids, histology and statistical analyses. Serum lipid profiles were determined for total cholesterol (Sigma Kit 352-20), triglycerides (Sigma 339-10) and HDL cholesterol (Sigma 352-3) using kit protocols (Sigma Chemical, St. Louis, MO). Samples were analyzed in duplicate and reaction mixtures were adjusted to 200 μL for the well volume of 96 well plates. Hearts and aortas from 40 mice (5 from each dietary treatment) were fixed in Karnovski's fixative and embedded in JB4 media (Polysciences, Warrington, PA) for sectioning, staining (Aparicio and Marsden 1969) and microscopic examination at magnifications of 40X and 400X. Identity of all sections was coded and unknown to the examiner. The Statistical Analyses System-General Linear Model (SAS GLM) program (SAS 1987) was employed for statistical computing with post-hoc analyses of statistical significance by Tukey's Multiple Range Comparison (HSD) (Steele and Torrie 1980). Results are reported as means ± SEM.

RESULTS

Food intake and weight gain. Growth curves for BALB/cByJ and C57BL/6J mice fed different types and levels of dietary fat for 16 wk are shown in Fig. 1. BALB/cByJ mice at 6–7 wk of age weighed more initially than C57BL/6J mice (19.0 vs. 16.1 g, respectively) at 6–7 wk of age and throughout the 16-wk feeding study ($P < 0.0001$). BALB/cByJ mice averaged 26.9 g compared with 22.2 g for C57BL/6 mice at the end of the study. Energy intake of BALB/cByJ mice was 53.8 ± 5.4 kJ/d during the 16-wk study, significantly more ($P < 0.005$) than that of C57BL/6J mice (45.5 ± 3.3 kJ/d). However, there were no consistently significant differences between strains in efficiency of energy utilization (data not shown). Mice fed diets with 20% oil consumed 54.3 ± 8.8 kJ/d compared with 45.1 ± 5.4 kJ/d for mice fed 4% oils, but no differences were observed in weekly energy efficiencies. Both C57BL/6J and BALB/cByJ mice fed 20% oil consumed more energy (24.2 ± 4.9 vs. 16.7 ± 5.8 kJ) in the 2-h refeeding period than their counterparts fed 4% oil ($P < 0.0001$), but there was no difference between strains.

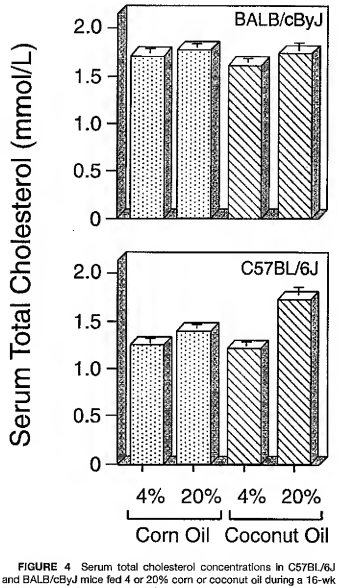


FIGURE 4 Serum total cholesterol concentrations in C57BL/6J and BALB/cByJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. Serum total cholesterol concentrations (mean ± SEM) are reported for 8–10 C57BL/6J and 8–10 BALB/cByJ mice fed each dietary treatment (except BALB/cByJ fed 20% coconut oil where $n = 7$). Main effects are reported in Table 2. Interactions between strain and oil level ($P < 0.0517$), strain and oil soure ($P < 0.0793$), and level and source ($P < 0.0801$) approached statistical significance.

TABLE 3

Pearson correlation analysis of strain, serum total cholesterol, HDL cholesterol and triglyceride concentrations in BALB/cByJ and C57BL/6J mice fed 4 or 20% corn oil or coconut oil for 15 wk[1,2,3]

	Strain	Oil level	Oil type	Total Chol[4]	HDL Chol	TG	ApoE/β-actin	Scd1/β-actin
Oil level	0.000							
Oil type	0.000	0.000						
Chol	−0.456***	0.302	0.079					
HDL Chol	−0.503	0.265*	−0.092	0.631***				
TG	−0.255*	0.340**	0.127	0.446***	0.252			
ApoE/β-act	0.121	0.052	−0.024	0.076	−0.198	0.105		
Scd/β-act	0.241*	−0.261*	0.152	−0.166	−0.237	−0.083	−0.080	
Lpl/GAPDH	−0.234	0.315	0.033	0.517**	0.360	0.278	0.173	−0.398

[1] Correlation coefficients (r).

[2] *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Significance was approached ($P < 0.06$) for HDL cholesterol, and triglyceride and Scd1/β-actin and Lpl/GAPDH ($P < 0.08$).

[3] $n = 60–80$, except for Lpl where $n = 19–24$.

[4] Chol, cholesterol; TG, triglyceride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Serum lipid concentrations. Fed BALB/cByJ mice had higher serum triglycerides ($P < 0.01$), HDL cholesterol ($P < 0.0001$) and total cholesterol ($P < 0.0001$) than did C57BL/6J mice (**Table 2**). The higher concentration of dietary oil also resulted in greater serum HDL cholesterol ($P < 0.01$), serum triglycerides ($P < 0.001$) and total cholesterol ($P < 0.001$) (Table 2). Serum triglyceride concentrations showed significant interactions between strain and oil source ($P < 0.001$) and between strain and dietary oil level and source ($P < 0.01$) (**Fig. 2**), but no interactions were significant for cholesterol (**Fig. 3**). Interactions tended to be significant between strain and dietary oil concentration ($P < 0.06$) and between strain and source ($P < 0.08$) and source and dietary oil level ($P < 0.08$) for serum total cholesterol concentrations (**Fig. 4**). Significant correlations between total cholesterol and HDL cholesterol ($r = 0.631$, $P < 0.001$) and triglycerides and total cholesterol were found (**Table 3**). There were no significant correlations between these serum lipid concentrations and body weight, total energy intake or energy eaten in the last meal (not shown).

Gene expression. Representative hybridization analyses are shown for hepatic stearoyl CoA desaturase and β-actin mRNA from individual BALB/cByJ (**Fig. 5A**) and C57BL/6J (Fig. 5B) mice fed one of the four diets with averages of Scd1/β-actin mRNA ratios for all in each treatment shown in **Figure 6A** (BALB/cByJ) and Figure 6B (C57BL/6J). Relative Scd1 levels were significantly higher ($P < 0.03$) in mice fed diets containing 4% of either oil even though mice eating 20% oil diets consumed more energy during their last meal. Scd1/β-actin mRNA ratios were significantly higher ($P < 0.04$) in C57BL/6J mice, but there were no significant differences in Scd1/β-actin between corn oil– and coconut oil–fed mice. There were no significant interactions between strain and dietary source or concentration. Significant differences in hepatic ApoE and heart lipoprotein lipase mRNA expression due to strain, oil level or oil source were not found (data not shown).

Pearson correlation analyses (Table 3) showed a weak inverse correlation ($r = −0.237$) between Scd1/β-actin and HDL levels that approached significance ($P < 0.08$). Lpl/GAPDH mRNA ratios were significantly correlated ($r = 0.517$, $P < 0.01$) with cholesterol levels and there tended to be a negative correlation ($r = −0.398$) approaching significance between Scd1/β-actin and Lpl/GAPDH mRNA ratios ($P < 0.08$).

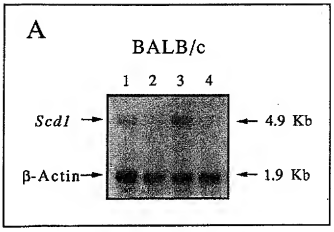
Histology. Forty hearts, examined for histological evidence of lesion development, showed no abnormalities except for three from BALB/cByJ mice (not shown). Thickening of the tunica intima in a coronary artery was observed in one BALB/cByJ mouse fed 4% coconut oil. One BALB/cByJ mouse fed 4% and one fed 20% corn oil showed swelling of endothelial linings of their aortas.

DISCUSSION

We analyzed growth, serum lipid values, and abundance of Scd1, Lpl, and ApoE mRNA (i.e., genetic factors) in BALB/cByJ and C57BL/6J mice fed different types and concentrations of dietary lipids, factors that influence disease severity in studies similar to Kirk et al. (1995). Our analyses were done in the absence of atherogenic chemicals added to a semipurified diet and without evidence of disease processes, which might influence gene expression or serum lipid concentrations in strains which differ in predisposition to diet-influenced diseases.

In our study, BALB/cByJ mice were significantly heavier than C57BL/6J mice initially at 6–7 wk of age and throughout the subsequent 16-wk feeding period, but the weight gain/energy intake ratios were the same regardless of strain, oil level or oil source. Accuracy of intake measurements was facilitated by feeding compressed food pellets to individually housed mice with weighing of uneaten particles. The difference in energy intake during the 2 h when the last meal was available for mice fed 20% oil (24.2 kJ) compared with those eating 4% oil (16.7 kJ) may have resulted from differences in energy, texture or ease of eating the high fat diet. There were no significant correlations between energy intake and serum lipid values or between energy intake and levels of ApoE, Lpl, or Scd1 mRNA.

BALB/cByJ mice had significantly higher concentrations of total cholesterol, triglycerides and HDL cholesterol than C57BL/6J mice, in agreement with other investigators (Lusis et al. 1989). The increases in total cholesterol, triglycerides and HDL cholesterol were qualitatively and in some cases quantitatively similar to those seen in these and other strains fed high fat diets with and without added cholesterol (Kirk et al. 1995, Srivastava 1996, Srivastava et al. 1991 and 1992)



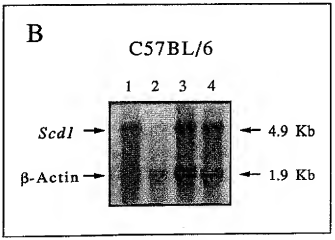


FIGURE 5 Effect of the level and type of dietary fat on hepatic stearoyl CoA desaturase (Scd1) expression in C57BL/6J and BALB/cBJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. RNA from four individual BALB/cByJ (*A*) and four C57BL/6J (*B*) mice fed 4% corn oil (lane 1), 20% corn oil (lane 2), 4% coconut oil (lane 3) and 20% coconut oil (lane 4) was separated on agarose gels and blotted. The resulting membranes were hybridized with radiolabeled Scd1 and β-actin probes as described in Materials and Methods. Similar data from 6–10 mice from each dietary treatment are summarized in Figure 4.

and atherogenic diets containing added cholic acid, cocoa butter and cholesterol (Hwa et al. 1992, LeBeouf et al. 1994, Nishina et al. 1993, Paigen et al. 1990, Warden et al. 1989). Saturated fat (coconut oil) significantly increased triglyceride levels in C57BL/6J but not in BALB/cByJ mice, and it did not alter total serum cholesterol or HDL cholesterol concentrations. Serum triglyceride concentrations were also affected by interactions among strain, oil level and oil source, demonstrating the importance of nutrient-genotype interactions. The variation in concentration in serum lipids for the same strains reported by different laboratories may be related to differences in diet composition because fatty acids and other ingredients vary among preparations of unpurified diets (e.g., Lardinos et al. 1989). Variations in serum lipid concentrations may also result from differences among studies in time between the last meal and the time of sampling (Kirk et al. 1995).

Heart lipoprotein lipase, hepatic stearoyl CoA desaturase, and hepatic apolipoprotein E mRNA abundances were analyzed as a follow-up of our previous isolation of Scd1 and ApoE in screens for diet-regulated genes. In our studies, abundance of hepatic ApoE and heart Lpl mRNA was unrelated to strain, fat level, fat source or energy intake in the last meal when mRNA levels were analyzed 2 h postprandially. Although others have shown strain differences in heart Lpl enzymatic activity (Ben-Zeev et al. 1983), Lpl mRNA levels are presumably not transcriptionally regulated by diet in hearts of mice (Kirchgessner et al. 1989) or rats (Erskine et al. 1993). Our Lpl data agree with this conclusion. Hepatic ApoE mRNA abundance was not regulated by level or source of oil. Others (Ishida et al. 1990) found strain (C57BL/6 and C3H/J)-specific differences in serum apolipoprotein E concentrations after-overnight food deprivation in mice fed unpurified vs. a highly atherogenic diet, suggesting that our results may be confounded because we analyzed expression 2 h postprandially. We previously found significant effects of fat level and time of eating on the regulation of Lpl (Paisley et al. 1996) and

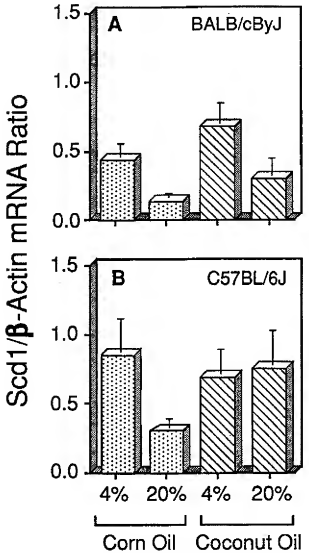


FIGURE 6 Hepatic stearoyl CoA desaturase (Scd1) expression in C57BL/6J and BALB/cByJ mice fed 4 or 20% corn or coconut oil during a 16-wk feeding study. Scd1 mRNA levels (representative results shown in Figure 3), normalized to β-actin mRNA, from 8–10 BALB/cByJ (*A*) and 8–10 C57BL/6J (*B*) mice of each dietary treatment were analyzed. Results are means ± SEM. Scd1/β-actin mRNA ratio was greater in C57BL/6J mice ($P < 0.04$) and higher in all mice fed 4% of either oil compared with those eating 20% of either oil.

ApoE in 10-wk-old BALB/cHnn mice denied food for 12 h compared with those that had eaten. Some diet-regulated genes differ in their expression related to ime of eating (LeBeouf et al. 1994, Paisley et al. 1996, Swartz et al. 1996) or age (Rao et al. 1989), showing that experimental variables in addition to dietary constituents may alter mRNA concentrations.

Significant differences in Scd1 mRNA abundance, a measure of transcription and mRNA stability, were observed between mice fed different levels of dietary fat when data from all BALB/cByJ and C57BL/6J mice were included in the analyses. Analyses of BALB/cByJ and C57BL/6J Scd1 regulatory regions indicated that differences in mRNA abundance were probably not due to sequence differences in the proximal 750 bp promoter region (data not shown). Sequences further upstream of this region were not analyzed. The results of this study confirm our findings showing fivefold greater abundance of Scd1 mRNA in livers of BALB/cHnn mice fed diets containing a low vs. high percentage of corn oil (Elliott et al. 1993, Paisley et al. 1996). Others found that Scd1 was regulated by saturation of dietary fat and by carbohydrates in livers of CD-1 male mice and rats (reviewed in Ntambi 1995), and more recently, by the peroxisome proliferator–inducing chemical, clofibrate (Diczfalusy et al. 1995). Hepatic Scd1/β-actin mRNA ratios tended to be higher in coconut oil–fed mice than in corn oil–fed mice. Diet composition or time between the last meal and time of killing may have been responsible for the observed differences between our studies and others (Ntambi 1992 and 1995). Scd1 expression showed significant strain differences, with BALB/c mice having lower expression than C57BL/6 mice, a strain susceptible to diet-induced atherosclerosis (Paigen et al. 1990), obesity and type II diabetes (Seldin et al. 1994, Surwit et al. 1995).

Pearson correlation analyses showed weak negative associations approaching significance between Scd1 abundance and HDL and Lpl abundance levels. Lpl abundance was significantly correlated with serum cholesterol concentrations. Because the concentrations and abundances of serum lipids and different mRNA are expected to be affected by multiple factors, it is not surprising that the correlations were weak. Although significant correlations show associations, it is not clear whether these interactions were directly linked or whether they correlated because of independent linkages with other common factors.

Candidate genes, which include those regulated by diet or other environmental factors (e.g., Berry et al. 1995), may be identified genetically if they map within disease or complex trait loci (e.g., QTL) and if they have some association with the disease process (Risch and Merikangas 1996). A small but growing number of disease QTL have been identified in humans and in laboratory animals (e.g., Berry et al. 1995, Risch et al. 1993, Seldin et al. 1994, and reviewed in Frankel 1995, Sim et al. 1995), but many possible loci remain unidentified because of the number of possible nutritional and genetic combinations yet to be analyzed (e.g., Patterson et al. 1991, Risch and Merikangas 1996). Consequently, genes not mapping to a QTL can not be excluded from participating in disease processes. ApoE maps to a region of mouse chromosome (Chr) 7 (Encylcopedia of the Mouse Genome 1996), which overlaps a Type I diabetic QTL (Risch et al. 1993), and its overexpression in transgenic mice reduced the hyperlipidemia associated with experimentally induced diabetes (Yamamoto et al. 1995). Neither Scd1 (Chr19) (Keller et al. 1994) nor Lpl (Chr8) (Encylcopedia of the Mouse Genome 1996) map to known disease QTL, although Scd1 can be misregulated by streptozocin in rats (Waters and Ntambi 1995) and experimentally induced hypertriglyceridemia in diabetic mice was decreased in transgenic mice overexpressing Lpl (Shimada et al. 1995). Additional experiments are required to test whether Scd1 or other diet-regulated genes are involved in the molecular mechanism of disease.

ACKNOWLEDGMENT

The authors thank Peter Imrey (Department of Medical Statistics, University of Illinois College of Medicine, Urbana, IL) for helpful discussions concerning statistical analyses.

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Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia

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Abstract Stearoyl-CoA desaturase (SCD) is expressed at high levels in several human tissues and is required for the biosynthesis of oleate (18:1) and palmitoleate (16:1). These monounsaturated fatty acids are the major components of phospholipids, triglycerides, wax esters, and cholesterol esters. Mice with a targeted disruption of the SCD1 gene have very low levels of VLDL and impaired triglyceride and cholesterol ester biosynthesis. In the HYPLIP mouse, a model of hyperlipidemia, there was a 4-fold increase in hepatic SCD activity, a 1.8-fold increase in the desaturation index, and a 2-fold increase in plasma triglycerides. We used the plasma ratio of 18:1/18:0 (the "desaturation index") as an in vivo measure of SCD activity in human subjects. In human subjects with triglycerides ranging from 0.3 to 20 mM, the desaturation ratio accounted for one-third of the variance in plasma triglyceride levels. A 2-fold increase in the desaturation index was associated with a 4-fold increase in plasma triglycerides. In human subjects exposed to a high carbohydrate diet, the desaturation index explained 44% of the variance in triglycerides. We propose that many of the factors that influence plasma triglyceride levels do so by converging upon the regulation of SCD activity.—Attie, A. D., R. M. Krauss, M. P. Gray-Keller, A. Brownlie, M. Miyazaki, J. J. Kastelein, A. J. Lusis, A. F. H. Stalenhoef, J. P. Stoehr, M. R. Hayden, and J. M. Ntambi. Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia. *J. Lipid Res.* 2002. 43: 1899–1907.

Supplementary key words metabolic syndrome • hyperlipidemia • monounsaturated fatty acids

The rate of de novo fatty acid synthesis is low in human populations that consume diets high in polyunsaturated fatty acids (PUFA) and cholesterol, known repressors of lipogenic gene expression (1). However, one lipogenic gene that is highly expressed in various human tissues even in the presence of cholesterol and PUFA-rich diets is stearoyl-CoA desaturase (SCD) (2).

SCD catalyzes the introduction of the first *cis*- double bond in the Δ9 position (between carbons 9 and 10) in several fatty acyl-CoA substrates. The preferred substrates are palmitoyl- and stearoyl-CoA, which are converted into palmitoleoyl- and oleoyl-CoA, respectively (3). Changes in activity of SCD in tissues are reflected in the composition of cellular phospholipids, cholesterol estersn and triglycerides.

SCD gene expression is highly regulated; it is very sensitive to dietary lipids (PUFAs, cholesterol, and vitamin A), hormones (insulin), developmental processes, temperature changes, thiazolidinediones, metals, alcohol, peroxisomal proliferators, and phenolic compounds (4). Consequently, genetic variability in numerous pathways might manifest itself in changes in SCD expression and thus have consequences on lipid metabolism.

The liver and adipose tissue are the principal sites of de novo lipogenesis. Both tissues have a high capacity to convert carbohydrate into fatty acids when glycolytic and lipogenic enzymes are induced and activated. Recently, the transcription factor sterol responsive element binding protein-1c (SREBP-1c) has emerged as a master regulator of these metabolic pathways (5). SREBP-1c activates the

Manuscript received 8 May 2002 and in revised form 1 July 2002.
Published, JLR Papers in Press, August 16, 2002.
DOI 10.1194/jlr.M200189-JLR200

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expression of genes encoding enzymes necessary for conversion of carbohydrate to fat (e.g., glucokinase, acetyl-CoA carboxylase, ATP-citrate lyase, glucose-6-phosphate dehydrogenase, and malic enzyme). SCD is exquisitely sensitive to SREBP-1c regulation (4, 6–8). The discovery that insulin stimulates the transcription of the SREBP-1c gene readily explains how insulin exerts a global stimulatory effect on lipogenesis (9).

Using a naturally occurring mouse model of SCD1-deficiency, as well as a gene-targeted SCD1-knockout mouse, we have shown that triglyceride and cholesterol ester synthesis are greatly reduced in the absence of this enzyme (10). The triglyceride phenotype cannot be reversed with dietary monounsaturated fat, suggesting that de novo synthesized monounsaturated fat is essential for triglyceride synthesis (10, 11). The SCD1-deficient animals produce very low levels of VLDL, suggesting that the rate of VLDL production might itself be influenced by SCD1 activity in vivo (10, 11).

The role of SCD in human lipoprotein metabolism has never been explored. Hypertriglyceridemia (HTG) syndromes are among the most common lipid disorders in humans. Although there is strong evidence that many of these syndromes are heritable (12–14), the genetics of these disorders is not well understood. It is most likely that HTG is a complex trait; i.e., the expression of the phenotype is influenced by multiple genes. In addition, the penetrance of HTG is affected by diet, insulin sensitivity, and obesity (15).

Given the strong correlation between the ability of cells to synthesize triglycerides and cholesterol esters, and the activity of SCD, we validated and applied a simple plasma marker of SCD activity, the ratio of plasma oleate to stearate (18:1/18:0 ratio, the "desaturation index"), to test the hypothesis that in vivo SCD activity accounts for a large fraction of the variation in plasma triglycerides in human subjects. In addition, we studied the desaturation index in human subjects exposed to a regimen known to raise serum triglyceride levels, high-carbohydrate diets. Our results support an important role of SCD in determining human serum triglyceride levels.

METHODS

Animals and diets

Asebia homozygous (ab^J/ab^J or $-/-$) and heterozygous ($+/ab^J$ or $+/-$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Wisconsin Animal Care Facility.

In this study, comparisons are made between the homozygous ($-/-$) and the heterozygous ($+/-$) mice since the latter are indistinguishable from normal mice. The generation of targeted $SCD^{-/-}$ mice has been previously described (11). Prebred homozygous ($SCD1^{-/-}$) and wild-type ($SCD1^{+/+}$) mice on an SV129 background were used. C3H mice and HcB19 mice were bred at UCLA by A. Jake Lusis and shipped to Madison, WI for analysis. These mice were housed in a pathogen-free barrier facility operating on a 12-h light/12-h dark cycle. At 3-weeks-of-age, these mice were fed ad libitum on laboratory chow diet (5001 test diet, PMI Nutrition International Inc., IN).

Plasma lipoprotein analysis. Mice were fasted a minimum of 4 h and sacrificed by CO_2 asphyxiation and/or cervical dislocation.

Blood was collected aseptically by direct cardiac puncture and centrifuged (13,000 *g*, 5 min, 4°C) to collect plasma. Lipoproteins were fractionated on a Superose 6HR 10/30 FPLC column (Pharmacia). Plasma samples were diluted 1:1 with PBS, filtered (Cameo 3AS syringe filter, 0.22 μm), and injected onto the column that had been equilibrated with PBS containing 1 mM EDTA and 0.02% NaN_3. The equivalent of 100 μl of plasma was injected onto the column. The flow rate was set constant at 0.3 ml/min. Five hundred microliter fractions were collected and used for total triglyceride measurements (Sigma). Values reported are for total triglyceride mass per fraction. The identities of the lipoproteins have been confirmed by utilizing anti-ApoB immunoreactivity for LDL and Anti-Apo A1 immnunoreactivity for HDL (not shown).

Fatty acid analysis. Total lipids were extracted from plasma according to the method of Bligh and Dyer (16). Heptadecanoic acid (Sigma) was added as an internal standard for the quantitation of fatty acids. The lipids were methylated and analyzed by gas-liquid chromatography on a capillary column coated with DB-225 (0.25 mm, 30 m length id, 0.25 μm; Agilent Technologies, Inc. Wilmington, DE). Colum temprature was kept at 70°C for 1min, then increased from 70°C to 180°C at a rate of 20°C/min and then to 220°C at a rate of 3°C/min, and was kept at 220°C for 15 min. 20:1n-9 and 20:1n-7 fatty acids were identified by comparison of retention times with authentic standards (Sigma, St. Louis, MO).

Familial combined hyperlipidemia patients. One hundred patients with hypertriglyceridemia were selected from a Dutch cohort of 32 families (299 subjects) with well-defined familial combined hyperlipidemia (FCH) (17).

Carbohydrate feeding study. Human subjects were selected from a combined group of 429 healthy, nonsmoking Caucasian individuals aged ≥20 who had participated in previous dietary intervention protocols as described (18, 19). All subjects had been free of chronic disease during the previous 5 years and were not taking medication likely to interfere with lipid metabolism. In addition, they were required to have plasma total cholesterol concentrations <6.74 mM (260 mg/dl), triglyceride <5.65 mM (500 mg/dl), resting blood pressure <160/105 mm Hg, and body weight <130% of ideal. Each participant signed a consent form approved by the Committee for the Protection of Human Subjects at E. O. Lawrence Berkeley National Laboratory, University of California, Berkeley, and participated in a medical interview. Fasting blood samples were obtained after 4–6 weeks in which subjects consuming high-fat/low-carbohydrate diets (carbohydrate 39–45% en) and low-fat/high-carbohydrate diets (carbohydrate 61–65% en) (1, 2). In all diets, ~50% of the carbohydrate was consumed as simple sugars.

Plasma lipid and lipoprotein measurements were performed as previously described (1). We wished to compare results in two subgroups of subjects selected on the basis of triglyceride responses to a high carbohydrate diet greater than 1 SD above or below the mean for the entire group (29.2 ± 77.9 mg/dl). Within each category, subjects were matched within 20% for basal triglyceride level on the low carbohydrate. As a result, mean levels of basal triglyceride were similar in the two groups, as were BMI and age. Statistical analyses were performed using Statview (SAS Institute, Inc.). Diet differences were analyzed by paired *t*-test within group correlations by Spearman correlations and between group differences by Mann-Whitney tests. Logistic regression analysis was used to assess determinants of triglyceride response category.

Relationship between SCD1 gene dosage and the desaturation index

We measured the ratio of oleate (18:1) to stearate (18:0) and of palmitoleate (16:1) to palmitate (16:0) in two mouse models of SCD1 deficiency. The asebia mouse has a naturally-occurring deletion encompassing most of the SCD1 gene (20). Homozygotes have no SCD1 expression and express residual desaturase activity of <10% of normal, due to the expression of SCD2. The 18:1/18:0 ratio was reduced about 50% in homozygotes relative to heterozygotes (Fig. 1A). The 16:1/16:0 ratio was reduced by about two-thirds (Fig. 1B).

We recently generated mice deficient in SCD1 through targeted disruption of the SCD1 gene (11). In these animals, there is a relationship between SCD1 gene dosage and both the respective C-18 and C-16 fatty acid ratios; heterozygotes have a triglyceride level intermediate between that of the wild type and that of the homozygous-null animals (Fig. 1C, D). The asebia mutation occurred in a BALB/c-derived mouse strain background (20). The *SCD1*-knockout mice are 129 × C57BL/6 F1 mice. The 18:1/18:0 ratio in the asebia heterozygotes is about double that of the *SCD1*-knockout heterozygotes. Interestingly, the triglyceride levels in the asebia heterozygotes are also twice the levels in the knockout heterozygotes. Thus, although strain background changes triglyceride levels, the quantitative relationship between the desaturation index and plasma triglycerides is preserved.

Deficiency of plasma VLDL in animals deficient in SCD1

Analysis of the lipoprotein profiles of both strains of *SCD1*-knockout mice revealed a striking reduction in VLDL triglycerides in both the asebia (Fig. 2A) and the knockout (Fig. 2C) mice. The cholesterol profiles revealed no significant differences between wild type and SCD1-deficient mice (Fig. 2B, D).

Correlation between desaturation index and plasma triglycerides in a murine model of familial combined hyperlipidemia

Castellani et al. identified a spontaneous mutation (termed "hyplip") in the HcB congenic strain background associated with a phenotype closely resembling familial combined hyperlipidemia (FCHL) (21). The mutation maps to mouse chromosome 1, in a region syntenic with a segment of human chromosome 1q21-q23 where familial combined hyperlipidemia has been mapped in a human population (14). The potential genetic parallel with FCHL suggested that it might be a useful model to explore the possible relevance of SCD to FCHL. Subsequent to these studies, the Hyplip gene was identified as thioredoxin interacting protein (*Txnip*) (22).

The HcB-19 strain was used to explore the relationship between hepatic SCD activity, the plasma desaturation index, and triglyceride levels. The triglyceride levels of the HcB-19 mice were about 2-fold higher than those of the C3H background strain (Fig. 3A). The SCD activity of liver microsomes was about 4-fold higher in the HcB-19 mice

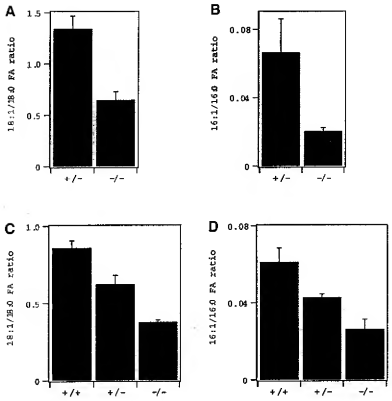

Fig. 1. The ratio of monounsaturated to saturated fatty acids in plasma (the desaturation index) is proportional to gene dosage in two mouse models of SCD1 deficiency. The steady-state levels of oleate (18:1), stearate (18:0), palmitoleate (16:1), and palmitate (16:0) were measured in fasting plasma collected from Asebia mice, a naturally occurring SCD1 deficient strain (A and B) and targeted SCD1 knockout mice (C and D). The data are expressed as the ratio of monounsaturated to saturated fatty acids for oleate/stearate (A and C) and palmitoleate/palmitate (B and D) and establish a "desaturation index" that is proportional to SCD1 gene dosage. The *P* values in the Asebia panels are derived from Student's two-tailed *t*-tests. The *P* values in the SCD1-knockout panels are derived from single factor ANOVA tests.

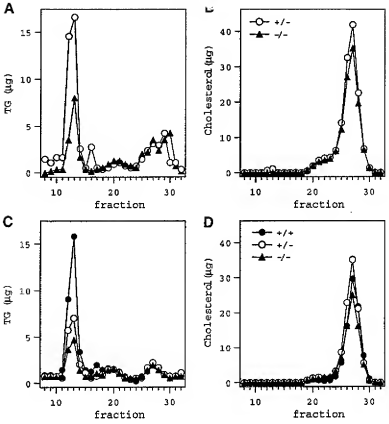

Fig. 2. Lipoprotein profiles in Asebia and SCD1 knockout mice demonstrate a selective loss of VLDL triglyceride. Plasma was collected from fasting Asebia (A and B) and SCD1 knockout mice (C and D) and lipoproteins isolated by fast performance liquid chromatography. Triglyceride (A and C) and cholesterol (B and D) levels were determined for each fraction and provide a distribution of these lipids among the various density fractions eluted from the column. The lipoprotein peaks for VLDL, LDL, and HDL are indicated.

than the controls (Fig. 3B). In this model, as in the SCD1 loss-of-function models described above, the desaturation index correlated with the SCD activity; the desaturation index was about 2-fold higher in the HcB-19 mice than in the C3H mice (Fig. 3C). There was only a small difference in the 16:1/16:0 ratio between the two strains (Fig. 3D). Despite the fact that these are C3H mice, the magnitude of the relationship between the desaturation index and

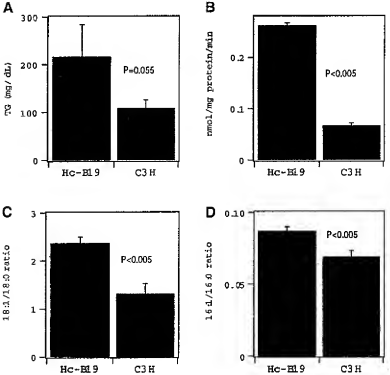

Fig. 3. A murine model for familial combined hyperlipidemia (FCHL) reveals elevated SCD1 activity, plasma triglycerides, and desaturation indices for plasma fatty acids. Fasting plasma from both mouse strains were used to determine triglyceride levels (A). SCD1 activity was determined in a liver microsomal fraction of normal (C3H) and hypertriglyceridemic (HcB19) mice (B). SCD1 desaturation index for oleate/stearate (C) and palmitoleate/palmitate (D).

triglyceride levels was similar to that of the other mouse strains we have assessed.

Correlation between desaturation index and plasma triglycerides in human subjects

We analyzed fasting plasma from 173 human subjects with plasma triglyceride levels ranging from 0.3 to 20 mM. One hundred of these subjects had familial combined hyperlipidemia (open circles, **Fig. 4**). The desaturation index correlated with triglyceride levels (Fig. 4A) and to a lesser extent, with HDL (Fig. 4B); the desaturation index explains 53% of the variation in plasma triglyceride and 17% of the variance in HDL. By contrast, there was no significant correlation between the 16:1/16:0 ratio and plasma triglycerides or HDL (Fig. 4C, D).

Responsiveness of desaturation index and plasma triglycerides to high-carbohydrate diet in human subjects

Human subjects were placed on a high carbohydrate diet (61–65% calories derived from carbohydrate) for 4–6 weeks. Out of 429 subjects, 20 whose triglycerides increased and 20 who did not show a triglyceride rise in response to the high-carbohydrate diet were chosen for measurement of the desaturation ratio. Prior to the start of the diet, the desaturation ratio explained 11% of the variance in plasma triglyceride (**Fig. 5A**). However, after consumption of the high-carbohydrate diet, the desaturation ratio accounted for 44% of the variance in plasma triglyceride (Fig. 5B). The correlation between these measurements was significant on both diets (Spearman $P < 0.0001$) in the group as a whole as well as in the subgroup with increased triglyceride on the high-carbohydrate diet ($P < 0.05$).

The plasma desaturation ratio increased significantly in both triglyceride response subgroups (**Table 1**). The increase was significantly greater, however, in the subgroup of subjects with increased versus decreased triglyceride in response to the high-carbohydrate diet. There was no significant correlation of the magnitude of triglyceride change

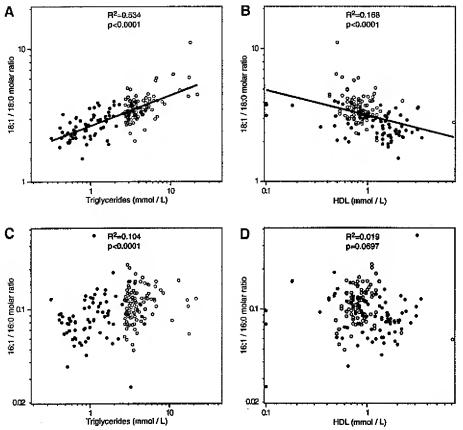

Fig. 4. The desaturation index correlates with plasma triglycerides and is inversely proportional to HDL levels in humans. Fasting plasma from 175 human subjects was used to determine triglyceride and HDL levels as well as determine the SCD1 desaturation index. The relationship between the 18:1/18:0 ratio and plasma triglyceride and HDL cholesterol is shown in A and B, respectively. The relationship between 16:1/16:0 ratio and plasma triglyceride and HDL cholesterol is shown in C and D, respectively. One hundred subjects (open circles) with familial combined hyperlipidemia had a mean plasma triglyceride of 4.6 ± 0.3 mmol/l and average HDL of 0.84 ± 0.02 mmol/l. The remaining subjects (closed circles) had a mean plasma triglyceride value of 1.3 ± 0.1 mmol/l and an average HDL of 1.36 ± 0.09 mmol/l. Three subjects shown in C were not included in the linear regression. For the normolipidemic group alone the correlation remained significant ($R^2 = 0.371$, $P < 0.001$), whereas for the FCHL group alone, it was not significant ($R^2 = 0.023$; $P = 0.13$).

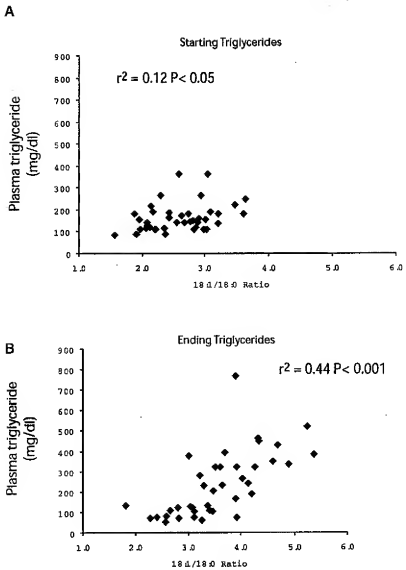

Fig. 5. Relationship between desaturation ratio and serum triglyceride concentration before (A) and after (B) 4–6 weeks on a high-carbohydrate diet. Subjects were placed on diets consisting of 61–65% calories derived from carbohydrate. Samples taken before and after the diet were analyzed for 18:1 and 18:0 fatty acids.

versus fatty acid ratio change in either group. However, both the desaturation ratio after the high-carbohydrate diet and the diet-induced change in this ratio were significant determinants of the triglyceride response category in logistic regression analysis ($P < 0.005$), accounting for 27% and 32% of the variance, respectively.

DISCUSSION

Despite 30 years of intense effort, the genetics of HTG syndromes is largely unsolved. The best-understood syndromes are those in which HTG results from defects in lipoprotein lipase (23). However, the most common forms of HTG involve overproduction of VLDL particles or the packaging of excess triglyceride on VLDL particles. With the exception of LDL receptor (LDLR) mutations (24, 25), no single gene defect has been shown to cause overproduction of lipoproteins in human subjects. Since the LDLR does not appear to be an agent in HTG and FCHL, new genes and perhaps new pathways must be identified.

The genetics of FCHL is especially challenging. As implied by the name, FCHL involves the transmission of elevated VLDL, LDL, or both within families. Until recently, it has been difficult to postulate regulatory mechanisms that might influence both cholesterol and triglyceride metabolism. Recent progress in understanding transcriptional regulation of lipogenic and cholesterogenic genes creates a new framework to bring to bear on these syndromes (26).

The SREBP genes, SREBP-1c and SREBP-2 regulate the lipogenic and cholesterogenic pathways, respectively (26). The abundance of the active forms of these transcription factors is regulated in two ways. First, the transcription of

TABLE 1. Lipid measurements (mean ± SEM) in groups with triglyceride increase or decrease on low versus high carbohydrate diets

Group	Triglyceride Increase (n = 20)		Triglyceride Decrease (n = 20)	
Dietary Carbohydrate	Low	High	Low	High
Triglyceride mg/dl	168.2 ± 15.0	368.3 ± 27.7[a,c]	160.0 ± 14.4	111.2 ± 10.1[a]
18:0/18:1 fatty acids	2.64 ± 0.13	4.10 ± 0.15[a,c]	2.56 ± 0.10	3.12 ± 0.15[b]

[a] $P < 0.0001$ versus low carbohydrate.
[b] $P < 0.01$ versus low carbohydrate.
[c] $P < 0.0001$ versus triglyceride decrease group.

SREBP-1c is regulated by insulin (9) and by the oxysterol-activated transcription factor, LXR (27). Second, the activation of the SREBP proteins, SREBP-1a, SREBP-1c, and SREBP-2, requires a proteolytic cleavage event that is inhibited by cholesterol (or an oxysterol) and polyunsaturated fatty acids (28). This web of regulatory mechanisms means that insulin sensitivity and its effect on insulin levels, dietary carbohydrate and fat, and cholesterol all interact to regulate SREBP-responsive genes. Given the range of potential genes that can conceivably be involved in these processes, it is not surprising that the genetics of these dyslipidemias is quite complex.

We have recently shown that SCD1 expression is essential and rate-limiting for the synthesis of triglycerides and cholesterol esters in mice (10). The supply of free fatty acids to the liver is rate limiting for the secretion of VLDL triglycerides (29). In rodents, high carbohydrate diets greatly enhance the synthesis and secretion of triglycerides by the liver (30). However, in the SCD1-knockout mice, carbohydrate feeding fails to induce triglyceride synthesis and secretion (11), implying that SCD1 represents a crucial bottleneck in this pathway.

In this manuscript, we show that SCD1 activity correlates with plasma triglyceride levels in several different mouse strains. We use these strains to validate the plasma desaturation index (the 18:1/18:0 ratio) as an in vivo surrogate of SCD activity and show that in human subjects, like the mouse strains, plasma triglyceride levels correlate with SCD activity. The desaturation ratio is closely related to plasma triglyceride levels in human subjects. This relationship extends throughout the normal range of triglyceride values. In addition, we found that a low-fat, high-carbohydrate diet induces an increase in the desaturation ratio, and that this increase is greater in individuals who exhibit a hypertriglyceridemic response to the diet than in those whose triglyceride is reduced. Moreover, although the magnitude of the changes in triglyceride and fatty acid ratios with diet were not significantly correlated, the increase in the fatty acid ratio was a significant determinant of triglyceride response category. Although the results are from a post hoc analysis and should be confirmed in other studies, they suggest that SCD activity is stimulated in conjunction with, and may contribute to, carbohydrate-induced lipemia. The finding that the mean 18:1/18:0 ratio increased in the group with a triglyceride reduction on the high carbohydrate diet suggests that other metabolic responses may have acted to reduce triglyceride production and/or increase triglyceride clearance in these individuals.

The desaturation ratio also explained a substantial proportion of the variance in triglycerides in subjects with hypertriglyceridemia. Although the HTG of these various subjects is undoubtedly due to various genetic and environmental causes, our most striking finding is that in most of the subjects, elevated triglycerides are accompanied by elevated SCD activity. Within the FCHL group (open circles, Fig. 4), the correlation between the desaturation ratio is lost ($R^2 = 0.023$, $P < 0.0001$ for the FCHL patients; $R^2 = 0.371$, $P < 0.0001$ for the non-FCHL patients). The lack of a significant correlation between the 18:1/18:0 FA ratio and triglyceride levels among FCHL subjects in our study (Fig. 4) may be because once triglyceride level is elevated in conjunction with increased SCD, the contribution of SCD to the overall variance of triglyceride levels is diminished. This would be the case, for example, if one were to study the relationship between cholesterol level and LDLR expression in people who already have a mutation in the LDLR.

We propose that there are numerous causes of HTG that converge upon and exert their triglyceride elevating effects through SCD. For example, HTG is frequently associated with insulin resistance. Recent studies suggest that in the liver, insulin resistance is selective; it involves impaired suppression of gluconeogenesis without an impairment in insulin-stimulated SREBP-1c gene transcription (31). Thus, the hyperinsulinemia that usually accompanies insulin resistance syndromes might lead to enhanced SREBP-1c expression and thus enhanced SCD expression. A failure to suppress SREBP-1c activation by cholesterol or polyunsaturated fatty acids might cause SCD activity to rise above the critical threshold that causes HTG.

In humans, there is recent evidence that adipose tissue might be the major source of the carbohydrate-derived fatty acids that end up as VLDL triglycerides (32). Therefore, it is possible that plasma triglycerides correlate with liver SCD in mice and with adipose tissue SCD activity in humans. Recent evidence from mouse (33) and human (34) studies shows that in obesity, the expression of SREBP-1c goes down in adipose tissue, and in mice and humans (35, 36) goes up in the liver (37). This redistribution in SREBP-1c gene expression orchestrates a shift in the lipogenic burden away from the adipose tissue (38). SCD expression is tightly linked to SREBP-1c expression (8). If SCD expression in the liver is more closely associated with VLDL production than is SCD expression in adipose tissue, then the shift in SREBP-1c expression from adipose tissue to liver (if it also occurs in obese humans) might form the basis for the relationship between HTG and obesity.

The rate of bile acid synthesis is correlated with the rate of VLDL triglyceride secretion in humans (39) and in animal models (40). Bile acid sequestrant therapy up-regulates cholesterol-7α-hydroxylase (CYP-7A1), the rate-limit-

ing enzyme in bile acid production, and leads to increased VLDL triglyceride secretion (41). Impaired bile acid absorption due to reduced expression of the intestinal bile acid transporter is also associated with increased plasma triglyceride levels (42). Overexpression of CYP-7A1 in hepatoma cells (43) or in transgenic mice also leads to increased VLDL secretion. Recently, Miyake et al. reported that in the transgenic mice overexpressing CYP-7A1, SREBP1 and SREBP2 were upregulated as were genes regulated by these transcription factors; SCD1 mRNA abundance was increased 4.7-fold (40). Together with the results presented herein, it is likely that the mechanistic link between bile acid production and triglyceride synthesis is through the regulation of SCD expression. This link likely involves the CYP-7A1 oxidation of sterol-derived ligands of LXR, a transcription factor that regulates SREBP expression (27).

A limitation in genetic studies of complex traits such as HTG is that variation at numerous gene loci can contribute to the same phenotype. If plasma triglyceride concentration is the sole phenotype used to carry out linkage studies, then the power to detect single major genes will be lost in populations with biologically significant variation at many genes that contribute to the same phenotype. One way to overcome this problem is to sub-divide families based on sub-phenotypes. For example, in the search for a major gene responsible for familial hypoalphalipoproteinemia, it was crucial to separate low-HDL families with demonstrable cholesterol efflux defects from those with normal cholesterol efflux (44). In the present case, we note that there are HTG individuals with apparently normal SCD activity and others who fit the linear trend of high SCD activity with HTG. The difference in SCD activity might therefore be a useful sub-phenotype that can be used to identify major genes for HTG and FCHL.

Because of the convergence of several types of HTG syndromes on SCD expression, we hypothesize that SCD activity might be rate-limiting for triglyceride production in a wide array of dyslipidemias. This, together with the lipid profiles of the SCD-deficient mice, suggests that SCD might be an attractive target for triglyceride-lowering drugs.

This work was supported by Xenon Genetics, Burnaby, BC, Canada and National Institutes of Health Grant HL56593.

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Loss of stearoyl–CoA desaturase-1 function protects mice against adiposity

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Contributed by Jeffrey M. Friedman, June 28, 2002

Stearoyl–CoA desaturase (SCD) is a central lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids, mainly oleate (C18:1) and palmitoleate (C16:1), which are components of membrane phospholipids, triglycerides, wax esters, and cholesterol esters. Several SCD isoforms (*SCD1-3*) exist in the mouse. Here we show that mice with a targeted disruption of the *SCD1* isoform have reduced body adiposity, increased insulin sensitivity, and are resistant to diet-induced weight gain. The protection from obesity involves increased energy expenditure and increased oxygen consumption. Compared with the wild-type mice the *SCD1*−/− mice have increased levels of plasma ketone bodies but reduced levels of plasma insulin and leptin. In the *SCD1*−/− mice, the expression of several genes of lipid oxidation are up-regulated, whereas lipid synthesis genes are down-regulated. These observations suggest that a consequence of *SCD1* deficiency is an activation of lipid oxidation in addition to reduced triglyceride synthesis and storage.

Stearoyl–CoA desaturase (SCD) is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids. It catalyzes the introduction of the *cis* double bond in the Δ9 position of fatty acyl–CoA substrates. The preferred desaturation substrates are palmitoyl–CoA and stearoyl–CoA, which are converted to palmitoleoyl–CoA (16:1) and oleoyl–CoA (18:1), respectively (1–4). These fatty acids are requisite components of membrane phospholipids, triglycerides, cholesterol esters, and wax esters (5–7). Effects on composition of phospholipids ultimately determine membrane fluidity, and the effects on the composition of cholesterol esters and triglycerides can affect lipoprotein metabolism and adiposity. SCD expression is sensitive to dietary factors including polyunsaturated fatty acids, cholesterol and vitamin A, hormonal changes (i.e., insulin and glucagon), developmental processes, temperature changes, thiazolinediones, metals, alcohol, peroxisomal proliferators, and phenolic compounds (3). High SCD activity has been implicated in a wide range of disorders including diabetes, atherosclerosis, cancer, obesity, and viral infection (3, 8–13).

The existence of multiple SCD isoforms in mice (6, 14–18) and rats makes it difficult to determine the role of each isoform in lipid metabolism. New insights into the physiological role of the *SCD1* gene and its endogenous products came from recent studies of the asebia mouse strains (*ab*^{J} and *ab*^{2J}) that have naturally occurring mutations in *SCD1* (17–19) as well as a laboratory mouse model with a targeted disruption (*SCD1*−/−) (6). We used these animal models to show that *SCD1*−/− mice are deficient in hepatic triglycerides and cholesterol esters (7, 20). The levels of palmitoleate (16:1) and oleate (18:1) are reduced, whereas palmitate and stearate are increased in the lipid fractions of *SCD1*−/− mice. On a high carbohydrate diet supplemented with triolein, the cholesterol ester levels are corrected but the triglyceride levels are not reversed to the levels found in the wild-type mouse (7).

Apart from the dramatic alterations in triglyceride and cholesterol metabolism, the *SCD1*−/− mice are considerably leaner than their wild-type counterparts. Here, we show changes in metabolic rate and in the expression of genes encoding enzymes involved in lipid metabolism.

Methods

Animals and Diets. *SCD1*−/− mice in SV129 background were generated and genotyped as described (5). The wild-type (*SCD1*+/+), heterozygous (*SCD1*+/−) and homozygous (*SCD1*−/−) mice are housed and bred in a pathogen-free barrier facility of the Department of Biochemistry (Univ. of Wisconsin, Madison) operating at room temperature in a 12-h light/12-h dark cycle. The breeding of these animals was in accordance with the protocols approved by the animal care research committee of the Univ. of Wisconsin. At 3 weeks of age, the mice were fed *ad libitum* a standard laboratory chow diet or a high-fat diet for 23 weeks. The high-fat diet contains 195 g/kg casein, 3 g/kg DL-methionine, 377 g/kg sucrose, 150 g/kg corn starch, 153 g/kg anhydrous milkfat, 10 g/kg corn oil, 1.5 g/kg cholesterol, 60.067 g/kg cellulose, 35 g/kg mineral mix AIN-76 (170915), 4 g/kg calcium carbonate, 10 g/kg vitamin mix Teklad (40060), 1.2 g/kg choline bitartrate, and 0.033 g/kg ethoxyquin (antioxidant).The weight of each mouse within each group was measured weekly; the data are presented as means ± SD (n = 8, $P < 0.001$). The glucose tolerance and insulin tolerance were determined as described (21).

Measurement of Oxygen Consumption. Gender matched *SCD1*−/− and wild-type littermates were investigated in indirect calorimeters as described (22). Oxygen consumption rate (VO_2) and CO_2 production rate (VCO_2) were continuously assayed over 4 consecutive 23-h periods, including 12 h dark (1800–0600) and 11 h light (0600–1700).

Gene Expression Analysis. RNA was isolated from livers of 10 individual 6-week-old female mice by using a standard method (23). Mouse genome U74A arrays were used to monitor the expression level of approximately 12,000 genes and expressed sequence tags (Affymetrix). Genes differentially expressed were identified by comparing expression levels in *SCD1*−/− and wild-type mice (24, 25). For Northern blot analysis, 20 μg of total liver RNA was separated on an 0.8% agarose/formaldehyde gel, transferred onto nylon membrane, and hybridized with cDNA probes for the corresponding genes.

Results

Reduced Body Weight in *SCD1*−/− Mice Fed a High-Fat Diet. Although the growth curves of male *SCD1*−/− mice were similar to those of wild-type siblings on chow diet, a high-fat diet revealed large differences in weight gain in both males (34.2 g vs. 39.5 g, $P < 0.01$, Fig. 1) and females (27.7 g vs. 31.9 g, $P < 0.05$).

Abbreviation: SCD, stearoyl–CoA desaturase.

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www.pnas.org/cgi/doi/10.1073/pnas.132384699

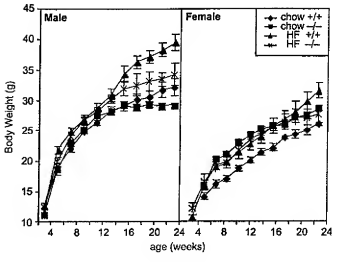
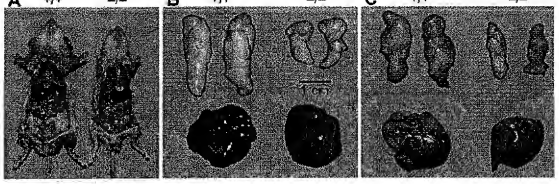


Fig. 1. Body weight of male and female wild-type and *SCD1*−/− mice fed a chow or high-fat diet.

Reduced Body Fat Mass in *SCD1*−/− Mice. On average, the *SCD1*−/− mice consumed 25% more food than wild-type mice (4.1 g/day vs. 5.6 g/day; $n = 9$, $P < 0.05$). Nonetheless, they were leaner and accumulated less fat in their adipose tissue (Fig. 2*A*). The epididymal fat pad mass was markedly reduced in male *SCD1*−/− relative to wild-type mice fed a chow diet (0.4 ± 0.1 mg vs. 0.8 ± 0.2; $n = 9$, $P < 0.05$; Fig. 2*B*) and a high-fat diet (1.0 ± 0.2 mg vs. 1.6 ± 0.2, $n = 12$, $P < 0.05$; Fig. 2*C*). The livers of the wild-type and *SCD1*−/− mice were grossly normal and of similar mass. In contrast, on a high-fat diet, the livers of the wild-type mice were lighter in color than those of the mutant mice (Fig. 2*C*), suggestive of hepatic steatosis. The masses of white adipose depots in *SCD1*−/− mice were globally reduced in mice on either the chow or the high-fat diet (Fig. 2*D*). The masses of other tissues, including brown adipose tissue, were not significantly altered. Thus, *SCD1*−/− mice were resistant to diet-induced weight gain and fat accumulation, despite increased food intake.

Increased Oxygen Consumption in *SCD1*−/− Mice. We carried out indirect calorimetry to investigate whether the resistance to weight gain is caused by increased energy expenditure. The *SCD1*−/− mice exhibited consistently higher rates of oxygen consumption (had higher metabolic rates) than their wild-type littermates throughout the day and night (Fig. 3*A*). After adjusting for allometric scaling and gender, the effect of the knockout allele was highly significant ($P = 0.00019$, multiple ANOVA, Fig. 3*B*).

Because the increase in O_2 consumption occurred during the fasting phase (daytime) as well as during the feeding phase, the animals are more active in oxidizing fat. Although ketone bodies were undetectable in plasma from either strain during postprandial conditions, β-hydroxybutyrate levels were much higher in



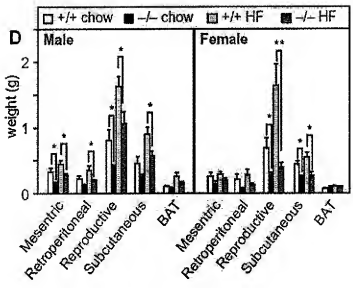


Fig. 2. (*A*) Abdominal view of the fat pad under the skin in 23-week-old male wild-type and *SCD1*−/− mice. (*B*) Epididymal fat pads and liver isolated from the wild-type and *SCD1*−/− mice on a chow diet. (*C*) Epididymal fat pads and liver isolated from the wild-type and *SCD1*−/− mice on a high-fat diet. (Bar = 1 cm.) (*D*) Fat pad weights from mice fed chow and high-fat diets.

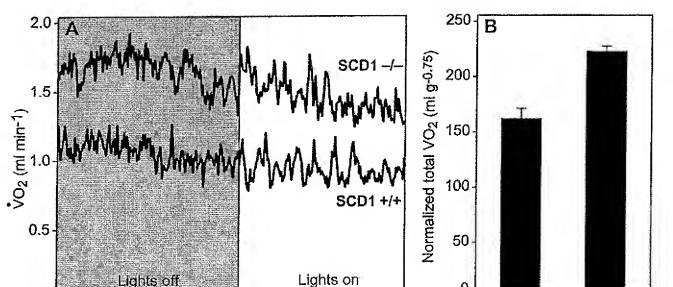


Fig. 3. *(A)* Metabolic rate and oxygen consumption of male mice on a chow diet. *(B)* Gender-adjusted, normalized total oxygen consumption over a 23-h period. Error bars denote SE.

the *SCD1*−/− mice after a 4-h fast (4.4 ± 0.6 mg/dl vs. 1.1 ± 0.7 mg/dl; $P < 0.001$), indicating a higher rate of β-oxidation in knockout mice. A similar but less dramatic difference was seen in females. These differences were also observed in mice on high-fat diet.

Increased Expression of Genes Involved in Fatty Acid Oxidation in *SCD1*−/− Mice. We used DNA microarrays to identify genes whose expression was altered in the livers of *SCD1*−/− mice. We identified 200 mRNAs that were significantly different between the livers of *SCD1*−/− and wild-type mice. The most striking pattern was seen in genes involved in lipogenesis and fatty acid β-oxidation. Lipid oxidation genes were up-regulated, whereas lipid synthesis genes were down-regulated in the *SCD1*−/− mice (Fig. 4*A*). Using the same RNA samples, the microarray data were verified with quantitative reverse-transcription–PCR using DNA primers that were designed for selected genes that showed differential expression (26). The results showed that the *PPARα*-target gene Fasting-Induced Adipocyte Factor (*FIAF*) was up-regulated in *SCD1*−/− mice ($P < 0.05$; Fig. 4*B*), whereas fatty acid synthase (*FAS*) was down-regulated ($P < 0.01$).

Northern blot analysis also supports changes in fatty acid oxidation and lipid biosynthesis. Probes for acyl-CoA oxidase (*ACO*), very long chain acyl-CoA dehydrogenase (*VLCAD*), and carnitine palmitoyltransferase-1 (*CPT-1*) indicate increases in β-oxidation (27, 28), whereas probes for *SREBP-1*, *FAS*, and glycerol phosphate acyl-CoA transferase (*GPAT*) point to a decrease in triglyceride biosynthesis (Fig. 4*C*).

Increased Insulin Sensitivity in *SCD1*−/− Mice. Reduced adipose tissue mass could either elicit insulin resistance or insulin sensitivity as demonstrated in several animal models (28). Fasting insulin levels were lower in the male *SCD1*−/− on chow diet (1.3 ± 0.3 ng/dl; $n = 7$) compared with wild-type mice (2.5 ± 0.9 ng/ml; $n = 7$). On a high-fat diet, insulin levels were similar between the two groups. Fasting glucose levels were similar between the *SCD1*−/− and wild-type mice. However, male and female *SCD1*−/− mice showed improved glucose tolerance compared with wild type (Fig. 5, $P < 0.05$). Thirty minutes after a glucose load, both male and female *SCD1*−/− mice tended to have lower fasting glucose levels (males: wild type, 345 ± 44 mg/dl; *SCD1*−/− mice, 202 ± 20, $n = 8$; females: wild type, 209 ± 20; *SCD1*−/− mice, 141 ± 9, $n = 5$). In addition, the glucose lowering effect of insulin was greater in the *SCD1*−/− mice than wild-type mice (data not shown). These data indicate that *SCD1*−/− mice have increased insulin sensitivity along with their loss of adiposity.

Discussion

These studies establish a critical role for SCD in the generation of body fat. The deletion of the SCD1 gene resulted in global changes in gene expression and altered metabolic activity that can account for the loss of body fat.

Genes encoding enzymes that participate in fatty acid oxidation were up-regulated in the *SCD1*−/− mice. *CPT-1*, *ACO*, *VLCAD*, and *FIAF* are known targets of *PPARα* (27, 28) and contain *PPARα* response regions in their promoters (28). Because *PPARα* mRNA is unchanged (data not shown), the up-regulation of enzymes of fatty acid β-oxidation in the *SCD1*−/− mice must be downstream of *PPARα* transcription. Thus, it is possible that loss of SCD1 function results in an increase in the concentration of a *PPARα* activator, perhaps a lipid ligand. The contents of saturated fatty acids (C16:0 and C18:0) are increased, whereas the contents of the polyunsaturated fatty acids of the *n*-6 and *n*-3 are not changed in the liver of the *SCD1*−/− mice (8, 10). One possible mechanism for our observations is that the saturated fatty acids induce the signal that activates the *PPARα* in the *SCD1*−/− mice, but this has yet to be determined. Alternatively the increased levels of C18:0- or C16:0-CoAs could inhibit acetyl-CoA carboxylase (*ACC*) through a well-known feedback mechanism; the resulting drop in malonyl-CoA can derepress *CPT-1*, resulting in increased transport of fatty acids into the mitochondria. Thus, the mechanism of increased lipid oxidation in the *SCD1*-deficient mouse could

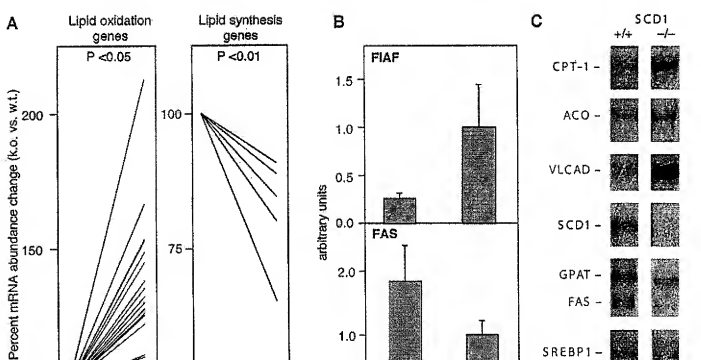


Fig. 4. (A) Expression levels of lipid oxidation (*Left*) and lipid synthesis (*Right*) genes between wild-type and *SCD1*−/− mice. (*B*) Quantitative reverse-transcription–PCR of *FIAF* and *FAS* gene expression, relative to wild-type mice. 18S RNA was used as a normalization control. (C) Northern blot analysis of lipid oxidation genes and lipid synthesis genes (*SREBP-1*, *FAS*, and *GPAT*) in the wild-type and *SCD1*−/− mice.

be caused by induction of *PPARα*-target genes as well enhanced availability of fatty acids for mitochondrial β-oxidation.

The *SCD1*−/− mice showed decreased expression in the liver of lipogenic genes *SREBP-1*, *FAS*, and *GPAT* (Fig. 4*C*). *SREBP-1c* is the main *SREBP-1* isoform expressed in the liver and regulates the expression of lipogenic genes (29). Insulin,

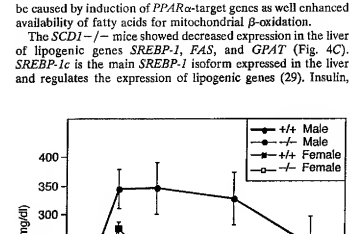


Fig. 5. Plasma glucose levels during the glucose tolerance test of male and female wild-type and *SCD1*−/− mice.

dietary carbohydrate, fatty acids, and cholesterol regulate *SREBP-1* gene expression and protein maturation (29, 30). Thus, the down-regulation of *SREBP-1* gene expression in the *SCD1*−/− mice could have numerous effects on various metabolic pathways regulated by *SREBP-1*. For instance the induction of *SREBP-1* by insulin and cholesterol greatly enhances the synthesis and secretion of triglycerides by the liver (31). However, in the *SCD1* knockout mice, carbohydrate feeding fails to induce triglyceride synthesis and secretion by the liver (7, 20). In addition, the *SCD1* deficiency attenuates triglyceride synthesis and very low density lipoprotein secretion in the *ob/ob* mouse (32), implying that *SCD1* represents a crucial bottleneck in triglyceride synthesis in the mouse.

In contrast to human subjects and several mouse models of lipodystrophy (33–36), the loss of adiposity in the *SCD1*−/− mice led to increased rather than decreased insulin sensitivity. In lipodystrophy, there is a redistribution in the lipogenic burden away from adipose tissue, leading to triglyceride accumulation in the liver and in skeletal muscle. Skeletal muscle triglyceride levels have recently been shown to strongly correlate with impaired insulin-stimulated glucose disposal. The reduction in muscle triglyceride content (M. Rahman, M.M., and J.M.N., unpublished data) in the *SCD1*−/− mice may contribute to increased insulin sensitivity observed in these mice.

Lipodystrophic *Crebbp* heterozygous null mice (37) have increased energy expenditure and unlike other lipodystrophic mouse models, increased insulin sensitivity. This has been attributed to increased plasma leptin levels. We measured plasma leptin to determine whether changes in levels of plasma leptin could account for the protection from weight gain, increased

energy expenditure and insulin sensitivity in the *SCD1−/−* mice. Plasma leptin was significantly reduced in the *SCD1−/−* mice relative to the wild-type controls (on chow diet: males, 5.0 ± 0.5 vs. 25.3 ± 5.5 ng/ml, $P < 0.01$; females, 5.1 ± 0.9 ng/ml vs. 11.1 ± 1.2 ng/ml, $P < 0.001$). A similar large difference was observed in mice on high-fat diet. Plasma leptin remained lower in *SCD1−/−* mice even after correcting for reduced fat mass. Thus, the *SCD1−/−* mouse does not resemble the *Crebbp+/−* mouse, because the protection from adiposity is present despite lower leptin levels. These data suggest that *SCD1* acts downstream of leptin, and predict that loss of SCD function would ameliorate the severe obesity observed in leptin-deficient *ob/ob* mice. Indeed, double mutant *asebia ob/ob* mice weighed significantly less than C57BL/6–*ob/ob* mice (32).

In conclusion, our studies have revealed that *SCD1* gene deficiency leads to resistance to diet-induced obesity, increased insulin sensitivity, and increased metabolic rate. Because leptin represses the expression of the *SCD1* gene and the *SCD1* deficiency normalizes the hypometabolic phenotype of the *ob/ob* mice (32), our results are consistent with *SCD1* being a target of leptin signaling, as suggested by the gene array studies of Soukas *et al.* (38) and confirmed by Cohen *et al.* (32). In addition, the expression of *PPARα* target genes of lipid oxidation were up-regulated in mouse liver of *SCD1−/−* mice, whereas those of *SREBP-1* target genes of lipid synthesis were down-regulated. The studies suggest that *SCD1* deficiency either directly or indirectly induces a signal that activates the *PPARα* pathway to partition fat toward oxidation and down-regulates *SREBP-1* expression thereby reducing lipid synthesis and storage. These metabolic changes recommend SCD as a promising therapeutic target for the many disorders associated with the metabolic syndrome.

We thank Dr. Jeffrey Peters for the cDNAs for *ACO*, *VLCAD*, *CPT-1*, and *PPARα*. We thank Mary Rabaglia for assistance with insulin measurements. We thank Yeonhwa Park for critical reading of this manuscript. This work was supported in part by National Institutes of Health Medical Scientist Training Program Grant GM07739 (to P.C.), National Institutes of Health Grants R01DK-41096 (to J.M.F.), HL-56593 and R01DK-58037 (to A.D.A.), and R01DK-62388 (to J.M.N.), and by Xenon Genetics, Inc. (to M.M.).

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1: Obesity (Silver Spring). 2007 Jul;15(7):1732-40.

Polymorphisms in the SCD1 gene: associations with body fat distribution and insulin sensitivity.

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OBJECTIVE: Obesity and insulin resistance are major risk factors for metabolic diseases and are influenced by lifestyle and genetics. The lipogenic enzyme, stearoyl-coenzyme A-desaturase (SCD), is related to obesity. Further, SCD1-deficent mice are protected against obesity and insulin resistance. We hypothesized that genetic polymorphisms in the SCD1 gene would be associated with obesity, insulin sensitivity, and estimated SCD activity in humans. RESEARCH METHODS AND PROCEDURES: The study population was 1143 elderly Swedish men taking part of a population-based cohort study, the Uppsala Longitudinal Study of Adult Men. Associations between single nucleotide polymorphisms and obesity (waist circumference and BMI), insulin sensitivity (assessed by hyperinsulinemic euglycemic clamp), and estimated SCD activity (fatty acid ratios) were analyzed using linear regression analysis. RESULTS: Subjects homozygous for the rare alleles of rs10883463, rs7849, rs2167444, and rs508384 had decreased BMI and waist circumference and improved insulin sensitivity. The rare allele of rs7849 demonstrated the strongest effect on both insulin sensitivity [regression coefficient (beta)=1.19, p=0.007] and waist circumference (beta=-4.4, p=0.028), corresponding to 23% higher insulin sensitivity and 4 cm less waist circumference. CONCLUSION: This study indicates that genetic variations in the SCD1 gene are associated with body fat distribution and insulin sensitivity, results that accord well with animal data. These results need confirmation in other populations with a larger sample size.

PMID: 17636091 [PubMed - indexed for MEDLINE]

1: Diabetologia. 2007 Nov 20 [Epub ahead of print]

Fatty acid desaturases in human adipose tissue: relationships between gene expression, desaturation indexes and insulin resistance.

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AIMS/HYPOTHESIS: Fatty acid desaturases introduce double bonds into growing fatty acid chains. The key desaturases in humans are Delta(5)-desaturase (D5D), Delta(6)-desaturase (D6D) and stearoyl-CoA desaturase (SCD). Animal and human data implicate hepatic desaturase activities in insulin resistance, obesity and dyslipidaemia. However, the role of desaturase activity in adipose tissue is uncertain. We therefore evaluated relationships between adipose mRNA expression, estimated desaturase activities (fatty acid ratios) in adipose tissue and insulin resistance. METHODS: Subcutaneous adipose tissue mRNA expression of D5D (also known as FADS1), D6D (also known as FADS2) and SCD was determined in 75 individuals representative of the study population of 294 healthy 63-year-old men. Desaturation indexes (product/substrate fatty acid ratios) were generated from adipose tissue fatty acid composition in all individuals. Insulin resistance was defined as the upper quartile of the updated homeostasis model assessment (HOMA-2) index. RESULTS: The relevant desaturation indexes (16:1/16:0, 18:1/18:0, 20:4/20:3 and 18:3/18:2) reflected expression of SCD, but not of D5D or D6D in adipose tissue. Insulin-resistant individuals had a higher adipose tissue 18:1/18:0, but not 16:1/16:0 ratio than insulin-sensitive individuals. Individuals with a high adipose tissue 18:1/18:0 ratio were 4.4-fold (95% CI 1.8-11.8) more likely to be insulin resistant [threefold (95% CI 1.1-8.6) after adjustment for waist circumference and plasma triacylglycerol]. In a multiple regression model predicting HOMA-2, the independent effect of the 18:1/18:0 ratio was borderline ($p = 0.086$). CONCLUSIONS/INTERPRETATION: Adipose tissue desaturation indexes of SCD reflect the expression of the gene encoding the enzyme in this tissue. Elevated SCD activity within adipose tissue is closely coupled to the development of insulin resistance.

PMID: 18030445 [PubMed - as supplied by publisher]